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UNIVERSITY OF ALBERTA

A STUDY OF INTRALYSOSOMAL AMINO ACID CONCENTRATIONS IN NORMAL, AND MUTANT HUMAN FIBROBLASTS

BY

DR. ROSE GIRGIS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF PEDIATRICS

EDMONTON, ALBERTA

SPRING 1991

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **A STUDY OF INTRALYSOSOMAL AMINO ACID CONCENTRATIONS IN NORMAL, AND MUTANT HUMAN FIBROBLASTS** submitted by **DR. ROSE GIRGIS** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

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Date: April 23 1991

TO MY HUSBAND, SAFWAT
MY SON, DANIEL
AND MY PARENTS, RAMSES AND KAMELIA

...WITH LOVE AND GRATITUDE

Abstract

An attempt to develop a method to measure the intralysosomal amino acid concentrations of normal and mutant human fibroblasts, is described. Cystinotic and I-cell disease fibroblasts were selected to test this method since they have previously been shown to have intralysosomal accumulation of cystine due to defective cystine lysosomal transport.

Normal, cystinotic, and I-cell disease fibroblast lines were grown in tissue culture, and lysosomal fractions were isolated on a self generating Percoll gradient. Subcellular fractionation of fibroblasts yielded two lysosomal marker enzyme (β-hexosaminidase) peaks (p= 1.049-1.054, and p= 1.096-1.107). Lysosomal membrane integrity was conserved to the extent of 75-85% in most granular fractions as judged by assaying β-hexosaminidase in the presence and absence of Triton X-100. The activities of marker enzymes succinate dehydrogenase for mitochondria, and 5'nucleotidase for plasma membranes were found in the buoyant fractions near the top of the Percoll gradient. Therefore, the heavy lysosomal fractions were mainly used in this study.

A novel approach was tried to determine the lysosomal free amino acid pool using a sensitive High Pressure Liquid Chromatography (HPLC) system. The advantage of this technique is that it allows the study of a whole amino acid profile in one tracing. To attain the required separation and sensitivity, the instrument is equipped with multiple solvent capability, an ion exchange column, and post column derivatization with ortho-phthalaldehyde (OPA) and fluorescence detection of the amino acid derivatives.

Amino acid analysis was performed in cystinotic, I-cell disease, and normal fibroblast homogenates and in their lysosomal fractions. In agreement with previous reports, the cystine content was found to be increased approximately ten fold in cystinotic fibroblasts compared to normal lines. Cystine was present in cystinotic lysosomal fractions, but was not detected in normal ones. Whereas, in I-cell disease fibroblast homogenates, cystine content varied from non detectable to slightly higher than control cell lines, and their lysosomal fractions showed only traces of cystine. The increased cystine content in cystinotic and I-cell lines was unique and did not reflect a generalized or specific increase in other amino acids content indicating that there are no other abnormal amino acid carriers.

The recovery of amino acids from fibroblast homogenates were found fairly reproducible, however intra lysosomal amino acid pool was found to be very variable, probably due to physiological heterogeneity of lysosomes and multiple experimental variables such as cell culture age and passage number, methods of sample preparation and storage temperature.

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Chapter one: Literature review

1. Introduction: Subcellular organelles

In higher organisms, there is no "typical" cell, as eukaryotic cells vary according to their locations. However, there are major intracellular compartments or organelles that are common to all eukaryotic cells, and perform specific functions.

The limiting membrane of every cell, the plasma membrane, is a continuous bi-layered sheet of phospholipid molecules and other complex lipids, about 4-5 nm thick, in which various proteins are embedded. These proteins serve a variety of functions; some are pumps, channels, or carriers for transporting specific molecules into and out of the cell.

The nucleus contains the main genome and is actively involved in DNA synthesis and repair, and in RNA synthesis; while RNA processing and ribosome synthesis occur in the nucleolus.

The surrounding cytoplasm consists of the cytosol and the cytoplasmic organelles where the metabolism of carbohydrates, lipids, amino acids, and nucleotides, as well as protein synthesis takes place. The endoplasmic reticulum (ER) is formed of flattened sheets, sacs, and tubes of membrane extending throughout the cytoplasm. It consists of "rough" ER and "smooth" ER. The former is formed of flattened sheets studded on its outer surface with ribosomes engaged in proteins synthesis. The smooth ER is more tubular and lacks the attached ribosomes, and its major function is lipid metabolism. The Golgi apparatus is a specialized part of the smooth ER consisting of stacked, membrane-bounded, flattened sacs which receive carbohydrates, lipids and proteins from the ER, modify, sort, and export them to a variety of intracellular destinations.

Mitochondria contain two concentric membranes, an inner and an outer one, separated by an intermembrane space. The inner membrane is characterized by its plate like invaginations, the"cristae". These organelles provide energy for the cell: they generate most of the ATP utilized to drive biosynthetic reactions that require an input of free energy. They are also involved in oxidation of carbohydrates and lipids, in urea and heme synthesis, and in the control of cytosolic (Ca^{2+}).

Peroxisomes are membrane-bounded vesicles, containing oxidative enzymes which generate and destroy hydrogen peroxide. The cytoskeleton of the cell, which is formed by microfilaments and microtubules, plays a role in the cell morphology and in the motility of both the cell and the intracellular components.

Finally, lysosomes are membrane-bounded vesicles, containing digestive enzymes which sequester intracellular organelles and degrade macromolecules and particles taken up from outside the cell by endocytosis. In addition to the major membrane-bounded

organelles described above, cells contain many small vesicles that act as carriers between organelles, as well as other vesicles that communicate with the plasma membrane in the secretory and endocytic pathways.

Lysosomes stand out as a unique class against all other subcellular organelles by their polymorphism and by the variety of processes, both physiological and pathological, in which they are involved. The study of the lysosomal system has uncovered a great deal of details. For the purpose of my study, I shall emphasize those aspects of lysosomes which are relevant to my research; the intralysosomal amino acid concentrations of normal and mutant human fibroblasts and lysosomal transport defects.

2. Lysosomes

The discovery of lysosomes is attributed to de Duve et al (1955). de Duve, who was awarded the Nobel prize, in 1974, in recognition for his discovery of lysosomes and his subsequent work on these and other cellular structures, described the early development of the field in the first of the series of books on lysosomes edited by Dingle and associates (1969a,b; 1973; 1975).

2.1 Definition of lysosomes

Although varying considerably in ultrastructure, lysosomes can be defined as membrane limited sacs, containing a variety of hydrolytic enzymes. This lysosomal concept rests essentially on two biochemical criteria: a) the association of acid hydrolases within special cytoplasmic particles and, b) the structure linked latency of the enzymes concerned (de Duve, 1983). The role of lysosomes in physiological and pathological conditions is thought to involve the digestion of both exogenous and endogenous substrates. Therefore, the name "lysosomes" was appropriately suggested by de Duve: Lysosomal enzymes break up their substrates by means of water, i.e. hydro (lyse) them, and are contained within particles ("somes" form the Greek soma).

2.2 The Formation and fate of lysosomes

Lysosomal enzymes are produced in the rough endoplasmic reticulum, then transfered to a specific region of the smooth endoplasmic reticulum located in the inner surface of the Golgi apparatus. These proteins are sorted by different receptors as they leave the Golgi apparatus in separate transport vesicles to meet in the endolysosome which already contains endocytosed material for digestion *(Fig 1.1)*. The substrates which are available for digestion originate by two main mechanisms: Heterophagy and Autophagy (Lazarus et al, 1975).

Heterophagy is a process whereby the cell engulfs extracellular material. This occurs either by phagocytosis or by endocytosis. Phagocytosis describes the uptake of large particles such as bacteria, protozoa, cellular debris, etc, and it is an activity carried out by cells of the reticuloendothelial system such as macrophages and polymorphonuclear leucocytes. In general, this process involves the formation of cytoplasmic extentions or pseudopodia around such particles producing a *phagosome* which later acquires lysosomal enzymes to become a *phagolysosome* (Sabatini and Adesnik, 1989)

Endocytosis refers to a variety of processes involving the internalization of extracellular substances such as hormones, growth factors, and nutrient-carrier proteins e.g. cholesterol incorporated in low density lipoprotein (LDL) particles (Goldstein et al, 1985), a process known as "receptor mediated endocytosis", and to the uptake of extracellular fluid and solutes what is known as "fluid phase endocytosis" or "pinocytosis". Kinetically receptor-mediated-endocytosis shows typical Michaelis-Menten kinetics as the system can become saturated. In contrast, pinocytosis is characterized by linear dependency on the concentration of the ingested solute. Endocytosis occurs when a portion of the cell plasma membrane surrounds extracellular fluid or solid particles forming a membrane bounded cytoplasmic compartment. In receptor mediated endocytosis the extracellular particles interact with specific cell surface receptors which mediate their interiorization. The ligand-receptor complexes become concentrated in small plasma membrane invaginations known as "coated pits". These pits form coated vesicles soon after pinching off into the cytoplasm (Pearse,1980). The membranes of these coated vesicles contain several proteins, of which the best characterized is "clathrin". The vesicles lose their coats as they fuse with membrane bounded tubulovesicular organelles known as endosomes. The current view is that a subclass of endosomes near the Golgi apparatus undergoes conversion into lysosomes by losing endosomal membrane components and by acquiring the necessary complement of lysosomal hydrolases (Griffiths et al, 1988).

On the other hand, autophagy is a process by which the cell sequesters its own cytoplasm and organelles forming *autophagosomes*. Fusion of the latter with *endolysosomes* initiates the digestion of its contents forming an *autophagolysosome*.

Lysosomes containing incompletely digested substrates, are known as *residual bodies*. These may either remain in the cytoplasm or may discharge their contents by exocytosis.

Figure 1.1 **Biosynthesis of lysosomes**

FIGURE OMITTED DUE TO COMPYRIGHT RESTRICTION

Source: Figure 8-71, p 461. Alberts et al: Transport of proteins from the Golgi apparatus to lysosomes. In: Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989. Lysosomes can be formed by three ways: phagocytosis, endocytosis, and autophagocytosis. These pathways meet at an intermediate compartment designated as endolysosome. Lysosomal hydrolases and membrane proteins are sorted by different receptors in the Golgi apparatus, then leave in separate transport vesicles to meet in the endolysosome.

2.3 Lysosomal membrane

Various studies have unveiled a great deal of knowledge about the morphology of the lysosomal membrane, its permeability to different molecules, and the factors affecting its structural integrity.

2.3.1 Morphology

Electron microscopic studies of the lysosomal membrane classify it with the thick type of biomembranes; i.e. 9 nm in thickness, resembling the plasma membrane and the membranes of maturing or transface Golgi apparatus (Yamamoto, 1963; de Duve and Wattiaux, 1966; Grove et al, 1968). A characteristic electron-lucent halo, present at the inner side of the membrane, has been described. It is a 7-10 nm layer which separates the membrane from the matrix (Daems and van Rijssel, 1961; Daems et al, 1969; Novikoff,

1973) and contains high concentrations of hydroxyl, vicinal glycol, and α-amino alcohol groups (Neiss, 1984).

The luminal surface of the membrane is protected from the effect of lysosomal proteases by complex oligosaccharides rich in sialic acid. These sialic acid containing residues can be detected by colloidal iron staining techniques (Henning et al, 1973). It was suggested that acting as immobilized polyanions, these sialic acid moieties might also create a Donnan potential for protons to maintain the acidic intralysosomal pH (Reijngoud and Tager, 1977). The majority of the lysosomal membrane proteins are exposed to the cytosolic space (Schneider et al, 1978); this might explain the digestion of lysosomal membranes when exposed to proteases from the outside (Henell et al, 1983a and b). The cytoplasmic surface of the lysosomal membrane possesses receptors that mediate the fusion of lysosomes among themselves or with phagosomes or endosomes to which lysosomal enzymes are provided. This surface was also found to interact with the cytoskeleton responsible for lysosomal movement (Mehrabian et al, 1984; Collot et al, 1984).

2.3.2 Lysosomal membrane permeability

In an attempt to briefly review the modes of transport of molecules across biological membranes, we find that, in general, there are two modes of transport: either simple diffusion or carrier mediated transport *(Fig 1.2)*. The latter can be either passive or active transport (Stryer, 1988). In simple diffusion, the transport is proportional to the concentration of solute. This is not commonly seen in biological membranes except in case of water (H_2O) which reaches equilibrium. The distinction between passive and active carrier mediated transport depends on the change in free energy of the transported material: active transport is coupled with an input of free energy, whereas, passive transport occurs spontaneously. Active transport processes can be driven by a variety of energy sources e.g. ATP, H^+ gradients, Na^+ gradients, and high potential electrons. ATP is one of the most common sources of free energy in active carrier mediated transport systems e.g. H^+ - K^+ ATPase transport system of the stomach, Na^+ - K^+ ATPase and Ca^{2+} ATPase systems found in animal cells, proton pumps found in endocytic vesicles, and F_O-F_1 H^+ ATPase system found in mitochondria. Active transport processes can be coupled with the flow of an ion down its electrochemical gradient: e.g. Na^+ gradients drive the active transport of glucose across the plasma membrane of intestinal and kidney cells. High potential electrons constitute a driving force for the proton pumping assemblies in oxidative phosphorylation.

Passive carrier mediated transport usually involves a protein in the membrane but no metabolic energy is required; a good example is well described in red blood cells. The latter contain an integral membrane protein known as the anion exchange protein which

mediates the exchange of HCO_3^- for Cl^-. There is a tight coupling between the effusion of one anion (HCO_3^-) and the entry of the other (Cl^-); this protein is defined as an antiporter and the obligatory nature of the exchange process results in electro-neutrality of the transport.

Another example of passive transport is seen in what are known as "Gap junctions". These are channels for passive transport clustered in the plasma membranes of opposed cells. They allow ions and most metabolic products such as sugar, amino acids, and nucleotides to flow between the interiors of contiguous cells. However, proteins, nucleic acids and polysaccharides are too large to cross these channels. These gaps are closed by high concentrations of Ca^+ and low pH (H^+); this feature likely serves to protect normal cells from traumatized or dying neighboring cells.

Figure 1.2 **Modes of transport across membranes: Simple diffusion and carrier mediated transport**

FIGURE OMIITED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 6-45, p303. Alberts et al: Membrane transport of small molecules. In: Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989.

Several studies have been performed to determine the permeability of the lysosomal membrane for the different end products resulting from the degradation of macromolecules. It was at first thought that the rates of penetration through the lysosomal membrane depended on variables such as molecular size, hydrophylic-hydrophobic nature, and in the case of ions, the ionic field strength. As a result, it was suggested that water soluble

substances such as monosaccharides and other polyols, amino acids, small peptides, and nucleosides, would cross from the lysosomal matrix to the cytosol and vice versa at rates that decrease with increasing molecular size and hydrophylicity (Lloyd, 1973; Reijngoud and Tager, 1977). The permeability of monovalent cations at 37°C was suggested to be as follows $Cs^+ > K^+ > Na^+ > H^+$, and that of anions: Thiocyanates > iodide > acetate > chloride > phosphate > bicarbonate > sulphate (Casey et al, 1978).

Recently, this classical view has been challenged by new evidence that describes the lysosomal membrane as being a selective permeability barrier between the lumen of the lysosomes and the cytoplasm. The passage of substances between both compartments was shown to be controlled or facilitated by carriers and transport systems found in the membrane. Recognition of those transport systems has occurred largely during the 1980's. The first evidence of a carrier mediated transport across the lysosomal membrane was demonstrated in the genetic disorder, nephropathic cystinosis. The evidence consisted of demonstration of saturation kinetics, stereospecificity, competition with structural analogues, temperature dependence, and countertransport. The excessive accumulation of cystine in this disorder was shown to result from a defective lysosomal cystine carrier (Gahl et al, 1982 a,b; Jonas et al, 1982). Since that time, lysosomal transport systems for cationic amino acids (Pisoni et al, 1985;1987a), large neutral and aromatic amino acids (Bernar et al, 1986; Stewart et al, 1988), small neutral amino acids (Pisoni et al, 1987b), and anionic amino acids (Collarini et al, 1988) have been described (*Fig 1.3*).

Lysosomes possess transport systems for sialic acid (Renlund et al, 1986 a;b), and vitamin B_{12} (Rosenblatt et al, 1985). Defects in those transport systems result in Salla disease and in a form of methylmalonic aciduria (cblF), respectively. In 1983, Maguire et al also provided evidence to support the existence of a lysosomal transport system for monosaccharides. A substrate specific facilitated diffusion for glucose has also been reported (Hales et al, 1984).

Another transport system involving a membrane associated enzyme, acetyl-CoA: α-glucosamide-N-acetyl transferase, has been described (Bame et al, 1985). This enzyme is necessary for the transfer of acetyl moieties from acetyl CoA in the cytoplasm, to acceptor glucosamine groups linked in terminal α-linkage to heparan sulfate molecules within the lysosomes. This acetylation step is essential for the degradation of heparan sulphate and, therefore, a deficiency in the transferase results in the accumulation of this mucopolysaccharide in the lysosomes. This disorder is known as Sanfillipo C syndrome (Klein et al, 1978). Finally, a nucleoside transport system has been recently characterized in human fibroblast lysosomes (Pisoni and Thoene, 1989).

Figure 1.3 **Provisional representation of the amino acid transport systems detected for human fibroblast lysosomes.**

FIGURE OMIITED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 1, p124. Christensen HN: Amino acid transport systems of lysosomes: Possible substitute utility of surviving transport system for one congenitally defective or absent. Biosc Rep 8(2): 121, 1988.

2.3.3 Structural integrity of the lysosomal membrane

The integrity of the lysosomal membrane can be assessed by measuring the extent of latency. This can be performed by assaying the activity of β-hexosaminidase, a lysosomal marker enzyme in the presence or absence of agents that disrupts lysosomes e.g. a detergent. The percentage of lysosomes that are broken is represented by the free β-hexosaminidase activity (measured in the absence of a detergent) expressed as a percentage of the total enzyme activity (measured in the presence of a detergent). Many factors such as freezing and thawing, vortexing, waring blender, inadequate osmotic protection, pH changes, high temperature, sonication, detergents, protease, and lecithinase have been found to disrupt lysosomes (de Duve, 1955). It has been shown that lysosomes kept at 0°C remained intact over a five hour period, while increasing incubation temperature (37°C) resulted in an increased rate of lysis of approximately 8% / hour. The lysosomal membrane was also found to break rapidly in pH < 4-5 and pH > 8, with the greatest stability being between pH 6 and pH 7.5 (Rome and Crain, 1981).

2.4 Lysosomal contents

Two different types of contents can be distinguished in the lysosomes (Pfeifer, 1987):

2.4.1 Intrinsic components: matrix and enzymes

2.4.2 Lysosomal food e.g. tracer substances, digestible contents, etc.

2.4.1 Intrinsic components

The intrinsic components consist mainly of the lysosomal matrix and lysosomal hydrolases.

2.4.1.1 Lysosomal Matrix

It was suggested that the lysosomal matrix is formed of non enzyme anionic substances which produce an acidic intralysosomal pH as a result of a Donnan equilibrium (Reijngoud et al, 1976), and that it is responsible for the protection against harmful substances such as silica and heavy metals.

2.4.1.2 Lysosomal Enzymes

The lysosomes possess a diverse array of enzymes most of which are hydrolases. The lysosomal enzymes found in different tissues vary according to the specific needs of individual tissues to digest specific substances. They are synthesized in ribosomes bound to the endoplasmic reticulum membrane, and the majority of them are converted to negatively charged glycoproteins containing N-linked and sometimes O-linked oligosaccharides chains. The nascent ribosome-associated chains contain cleavable N-terminal signals that lead to co-translational passage of the polypeptide into the lumen of the endoplasmic reticulum (Erickson and Blobel, 1979; Rosenfeld et al, 1982; Proia and Neufeld, 1982). The passage of this polypeptide across the endoplasmic reticulum membrane is paralleled by the acquisition of N-linked high mannose oligosaccharide chains and, by the cleavage of the signal sequence.

The lysosomal polypeptides undergo certain modifications which introduce in their oligosaccharide chains mannose-6-phosphate (Man-6-P), the lysosomal marker. The latter distinguishes lysosomal from secretory glycoproteins and addresses them to their lysosomal destination. In the cis Golgi, this recognition marker is generated through two sequential reactions catalyzed by a phosphotransferase and a phosphodiesterase, respectively. A defect in the enzyme N-acetylglucosaminyl-1-phosphotransferase which catalyses the first step results in the disorders known as I-cell disease (ML II) and Pseudo-Hurler-Polydystrophy (ML III). In these disorders, the cells are deficient in their ability to

equip the newly synthesized enzymes with the Man-6-P marker necessary for their targeting to lysosomes (Kaplan et al, 1977). It is believed that the phosphotransferase enzyme contains a site that recognizes a specific feature of the lysosomal polypeptide and positions it in proximity to the active site where modification of the mannose residue takes place.

Two types of Man-6-P receptor molecules, MPR^{CI} and MPR^{CD}, have been purified by affinity chromatography of solubilized cellular membranes on matrices containing immobilized ligands (Sahagian et al, 1981; Hoflack and Kornfeld, 1985). These receptors differ in their binding properties and in their divalent cation requirements. It was shown by immunocytochemistry that MPR^{CI} receptors are only found in coated vesicles, membranes of the Golgi apparatus and endolysosomes but not in mature lysosomes. Their affinity for the ligands is pH dependent. They bind strongly to lysosomal hydrolases bearing the marker (Man-6-P) at neutral or slightly acidic pH, and release them quantitatively at the high acidic intralysosomal pH.

In I-cell disease, the lysosomes in some cell types such as hepatocytes, Kupffer cells, and leucocytes were found to contain a normal complement of lysosomal enzymes (Owada and Neufeld, 1982; Waheed et al, 1982). This implies that alternate pathways may exist for directing hydrolases to lysosomes. The nature of these Man-6-P independent pathways is unknown. It was suggested that lysosomal enzymes which are present at high concentrations in the serum of ML II and ML III patients could be internalized through other systems which recognize other carbohydrate moeities such as galactose, N-acetylglucosamine, mannose, and L-fucose present in them (Waheed et al, 1982). It has also been reported that two lysosomal enzymes, β-glucocerebrosidase and acid phosphatase, are present at nearly normal levels in ML II and ML III fibroblasts. It is believed that membrane associated lysosomal enzymes, such as β-glucocerebrosidase, reach lysosomes through an unknown pathway which does not require phosphorylation (Granger et al, 1985; Barriocanal et al, 1986). However the normal acid phosphatase content in I-cell disease is an interesting feature since in normal fibroblasts its targeting to lysosomes appears to be Man-6P-dependent (Lemansky et al, 1985). Increased stability of this enzyme in lysosomes of I-cell fibroblasts might explain its accumulation (Nolan and Sly, 1989).

Lysosomal enzymes are all acid hydrolases optimally active near pH 5 which is maintained within the acidic lysosomal matrix. Certain enzymes seem to be primarily membrane bound (an esterase may be found on the outside of intact lysosomes) and are fully expressed in intact lysosomes. However, in vitro the majority of lysosomal enzymes are latent, that is to say, the lysosomal membrane must be disrupted to release them so that

they can react with their substrates in solution. This is termed "structure linked latency" (Dean, 1977).

2.4.2 Lysosomal food

The lysosomal food consists of material taken up into lysosomes, be it digestible or indigestible. For indirect visualization tracer substances such as haemoglobin (Goldfischer et al, 1970), peroxidase (Mori and Novikoff, 1977), and cytochrome C (Kiesewetter and Kugler, 1985) have been used; these are usually taken up by endocytosis into the lysosomal compartment. Other tracers can be directly identified by electron microscopy due to their high electron density i.e. compounds containing metals or due to their particular shape and structure e.g. latex particles (Daems et al, 1969). Ions of metals such as iron and manganese are found normally in lysosomes at higher concentrations than in other organelles; this can be explained by the fact that lysosomes sequester such ions to protect the cell form their deleterious effects.

A lysosomal amino acid pool has been identified (Harms et al, 1981). Lysosomes, isolated from rat liver, were shown to have significant intralysosomal pools of threonine, serine, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, and arginine. These amino acids are transported across the lysosomal membrane into the cytosol, where they can re-enter the metabolic pathways of the cell.

2.5 Intralysosomal pH

Published values for intralysosomal pH vary from < 4 to as high as 6.5 depending on the method of pH measurement. In vitro, the internal pH of lysosomes was found to be about one unit lower than the external medium (Reijngoud and Tager, 1973), whereas in vivo, the pH gradient across the lysosomal membrane is believed to be more than two units (Reijngoud and Tager, 1977; Ohkuma and Poole, 1978). It was controversial for a long time whether this low intralysosomal pH reflected an equilibrium of protons (Donnan type equilibrium) or an energy dependent mechanism, (active transport). According to the first mechanism, a pH difference across the lysosomal membrane is brought about by the presence of fixed negatively charged groups within the lysosomes (Reijngoud and Tager, 1977; Hollemans et al, 1979; 1980). However, recently the concept of an ATP-dependent lysosomal proton pump (Mego et al, 1972; Mego, 1979) has become widely accepted (Reeves and Reams, 1981; Reeves, 1984; Mellman et al, 1986). The lumen is maintained at an acidic pH by a H^+ pump in the membrane that utilizes cytoplasmic ATP *(Fig 1.4)* and functions in an electrogenic (Ohkuma et al, 1982; Harikumar and Reeves, 1983) or electroneutral (Schneider, 1983) manner, and operates most effectively in the presence of

chloride ions. The lysosomal pump strongly resembles other proton ATPases in its sensitivity to inhibitors.

Figure 1.4 **Intralysosomal pH**

FIGURE OMITTED DUE TO COPYRIGHT RESTRICTIONS

Source: Figure 8-69, p459. Alberts et al: Transport of proteins from the Golgi apparatus to lysosomes. In: The Molecular Biology of The Cell. Lackenbach L, Cobert S (eds), Garland Publishing Inc., New York, 1989. The intralysosomal lumen is maintained at an acidic pH ~ 5 by a H^+ pump in the membrane that uses the energy of ATP hydrolysis to pump H^+ into the vesicle

2.6 Metabolic pathways in lysosomes

In 1977, Barrett and Heath reviewed the properties of about 50 known lysosomal enzymes which included proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases, and sulfatases. These enzymes were classified by Barrett, in 1984, according to their contribution to the catabolic pathway into four classes: The"proteolytic", "glycanolytic", "nuclease", and "lipolytic" *(Table 1.1)*.

Table 1.1 **The classification of the enzymes involved in the lysosomal catabolic pathways**

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 1, p900. Barrett A: Proteolytic and other pathways in lysosomes. Biochem Soc Trans 12: 899, 1984

2.7 Heterogeneity of lysosomes

The heterogeneity of lysosomal morphology contrasts with the relatively uniform structures of most other cellular organelles. Density gradient subcellular fractionation of human diploid fibroblasts on Percoll-colloidal silica, separates two populations of particles containing acid hydrolases which can be considered lysosomal. The denser peak (density 1.11) is relatively free from contaminants and the more buoyant peak (density 1.04) sediments with other organelles such as mitochondria, endoplasmic reticulum, Golgi and plasma membrane. The activities of the following enzymes are used as markers for subcellular organelles: 5'nucleotidase (plasma membrane), succinate-p-iodonitrotetrazolium violet reductase (mitochondria) and β-hexosaminidase (lysosomes).

It has been demonstrated by both biochemical and ultra-structural methods that these two distinct populations did not appear to be due to artifactual trapping of one population by the other nor to release and redistribution of soluble enzyme. Both populations also showed latency. The origin of the buoyant and dense lysosomal organelles was suggested to be the GERL network[1] and the residual bodies, respectively (Rome et al, 1979). Another classification of lysosomes was reported: primary and secondary lysosomes. The term "primary lysosome" was used to designate lysosomes which contain the hydrolytic enzymes and have not yet acquired the substrate for digestion, that is they have not yet participated in a digestive event. These are in the form of small vesicles containing mannose-6-phosphate receptor molecules and carry newly synthesized hydrolases from the Golgi apparatus to endosomes undergoing transformation into lysosomes. The term "secondary lysosomes" was used to describe those lysosomes that have received substrates subsequent to endocytosis or autophagy. They are acid phosphatase rich vacuoles, 400-600 nm in diameter, containing hydrolases which have participated in or are engaged in a digestive event (Sabatini and Adesnik, 1989).

It is important to note that in fact the heterogeneity of lysosomes also reflects the character of material being digested within the lysosomes as well as the wide variety of digestive functions mediated by acid hydrolases. This polymorphism has almost become a hallmark by itself and for this reason, lysosomes are viewed as a heterogenous collection of distinct organelles whose common feature is a high content of hydrolytic enzymes.

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[1] The name GERL was chosen because of the location of the organelle on the concave (trans) aspect of the Golgi complex, being of an anatomical connection to the Endoplasmic Reticulum and of an apparent role in the genesis of Lysosomes.

3. *Lysosomal Disorders*

Two groups of deficient lysosomal functions have been described: deficiencies of lysosomal enzymes resulting in what is known as "lysosomal storage diseases", and defective egress of products from the lysosomes resulting in so-called "lysosomal transport defects".

3.1 Lysosomal storage diseases

In general, the digestion of macromolecules in the lysosomes is a stepwise process: each step is catalysed by a specific enzyme, and the product of one step forms the substrate for the next reaction in the digestive pathway. Therefore, deficiency of a lysosomal enzyme leads to the accumulation of its substrate(s) in the lysosomes. The storage of these undegraded molecules may result in cell, tissue, or organ dysfunction causing specific clinical syndromes. In man, more than 30 hereditary disorders have been described in which one or more lysosomal enzyme is defective (Tager et al, 1984).

In general, typical clinical features include: psychomotor retardation, coarse facial features, organomegaly, dysostosis multiplex, abnormal joint mobility, and corneal clouding. Simple enzyme assays are available for the diagnosis of the different enzyme deficiencies in peripheral leucocytes and or fibroblasts. Diagnosis is also suggested by the presence of increased urinary excretion of abnormal macromolecules. The genes for several of these enzymes have been cloned and mapped to specific chromosomes *(Table 1.2)* and different mutations have been described.

The deficiency of lysosomal enzymes can be due to several mechanisms *(Table 1.3)*: a) the precursor of the enzyme may not be synthesized e.g. α-Glucosidase deficiency in Pompe disease, early onset form, b) normal amounts of the precursor are synthesized but rapid degradation of the enzyme in lysosomes may occur e.g. Arylsulfatase A deficiency in Metachromatic Leukodystrophy, late onset form, c) the precursor may lack the mannose-6-phosphate recognition marker e.g. Deficient targeting of lysosomal hydrolases to lysosomes in I-cell disease, d) the precursor may have altered physiochemical and / enzymological properties e.g. Pseudodeficiency of Arylsulfatase A, e) the enzyme may be degraded due to the absence of a protective protein required for its stabilization e.g.β-Galactosidase deficiency in Galactosialidosis, f) the deficiency may result due to the absence of a factor required for enzymic activity e.g. absence of factor required for hydrolysis of sulfatide by Arylsulfatase A in a variant form of Metachromatic dystrophy (Tager et al, 1984).

Lysosomal storage diseases are mainly autosomal recessive genetic disorders except for Hunter's syndrome (Iduronate sulfatase deficiency) and Fabry's disease (α-Galactosidase deficiency) which are X-linked.

Table 1.2 **Chromosomal locations of hydrolase genes in humans as determined chiefly from investigations of storage diseases**

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 6.5, p 350. Holtzman E: Extensive release. Excessive storage. In: lysosomes. Siekevitz (ed.), Plenum Press, New York, 1989.

Table 1.3 The defects in human lysosomal storage diseases.

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

Table 1.3 (cont)

TABLE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Table 6.3, p 346. Holtzman E: Extensive release. Excessive storage. In: Lysosomes. Plenum Press, New York, 1989.

3.2 Lysosomal Transport Disorders: Cystinosis, Sialic Acid Storage Disorders, and an Inborn Error of Vitamin B_{12} Metabolism (cblF)

To date, lysosomal transport disorders include only three known diseases: Cystinosis, Salla disease, and one type of methylmalonic aciduria due an inborn error of vitamin B_{12} metabolism (cblF).

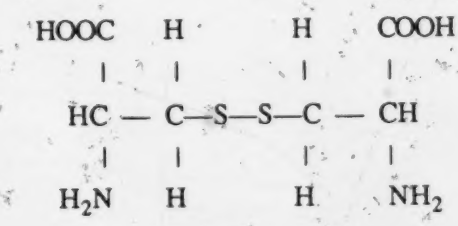
3.2.1 Cystinosis

In this review, emphasis is put on the investigations performed in cystinosis as cystinotic fibroblasts were used in this study, whereas the other two disorders are only briefly described.

3.2.1.1 Definition

Cystinosis is a rare autosomal recessive metabolic disorder, characterized by excessive intralysosomal accumulation of cystine (Schulman et al, 1969). Three forms of the disease have been described: nephropathic cystinosis, benign cystinosis, and intermediate (late onset) cystinosis (Kroll and Lichte, 1973). Patients with nephropathic cystinosis die in their first decade of life due to kidney disease, whereas those with the benign form have abnormal deposits of cystine in many organs but do not develop renal dysfunction. Patients with intermediate cystinosis have, together with the abnormal cystine deposition, a later onset of renal dysfunction.

Cystine is the disulfide of the amino acid cysteine. In the presence of oxygen, cysteine is rapidly oxidised to cystine.



Cystine

Cystine has a molecular weight of 240.3; its two carboxyl groups and its two amine residues have pK_a's of <1, 1.7, 7.48, and 9.02, respectively; its net charge at physiological pH is zero and its pI is 4.60 (Meister, 1965).

3.2.1.2 Localization of stored cystine in cystinosis

The plasma and urine levels of cystine and the intestinal absorption of cystine in cystinotic patients were found to be normal (Seegmiller et al,1968), indicating that the cystine accumulation is an intracellular phenomenon.

The localization of the stored cystine in the lysosomes was determined in several ways: Schneider et al (1967a;b) demonstrated by subcellular fractionation of cystinotic fibroblasts that the bulk of stored cystine co-sedimented in the granular fractions with the lysosomal enzyme acid phosphatase. In 1968, Patrick and Lake showed, by the electron microscopic study of a cystinotic lymph node, that the deposited cystine crystal was surrounded by acid phosphatase staining material all within an intact limiting membrane. In another study, Schulman et al (1970) described the lysosomal site of crystalline cystine deposits in cystinosis by ferritin uptake. Such evidence and many other studies have confirmed the lysosomal localization therefore, the following step was to define the underlying defect in cystinosis.

3.2.1.3 Source of accumulated cystine in cystinotic lysosomes

It was suggested that the possible sources of cystine in the lysosomes of cystinotic cells might include phagocytosed cystine, de novo synthesis via the cystathione synthetase-cystathionase pathway, glutathione degradation, and protein degradation.

Phagocytosis of preformed crystals accounting for intralysosomal cystine accumulation was ruled out based on earlier reports showing that the cystine crystals were absent in both urine and extracellular fluid and that they were soluble in plasma (Seegmiller et al, 1968).

Crawhall et al (1977) found that incubating cystinotic fibroblasts with labelled cystathionine or methionine did not result in the recovery of labelled cystine. De novo cystine synthesis through the cystathione β-synthetase pathway, therefore, did not appear to contribute to the cystine that accumulates in the cystinotic cells.

Thoene et al (1977) demonstrated that (^{35}S)cystine accumulated only when the protein pool was labelled and not when the glutathione pool alone was labelled .They also showed that when cystinotic fibroblasts were depleted from cystine by cysteamine and grown in a cystine deficient medium, the lysosomal cystine reaccumulated from the degradation of endogenous proteins. In another study, it was found that the lysosomal cystine content varied directly with the concentration in the growth medium of cystine rich protein, bovine serum albumin (Thoene and Lemons, 1980).

3.2.1.4 The basic defect in cystinosis

The investigation of the underlying defect in cystinosis was attempted by studying several possibilities. Oshima et al (1976) showed that cystine can reaccumulate in cystinotic cells even in the absence of extracellular cystine; therefore a defective plasma membrane transport accounting for cystine accumulation was ruled out. Another important possibility was abnormal cystine reducing systems. Data from cystinotic fibroblasts confirmed that there were normal amounts of cystine reductase, glutathione reductase, and cysteine glutathione thioltransferase (Kaye and Nadler, 1975). Several enzyme activities have been investigated: cystinotic γ- glutamyltranspeptidase activity was reported normal (Patrick et al, 1979), but arylsulphatase B (Furusho et al, 1971) and β- galactosidase (Scardigli et al, 1977) were found to be reduced in cystinotic fibroblasts. None of the above enzyme findings have been reported by more than one laboratory. Because the cytoplasmic catabolic pathways for cystine appeared normal in cystinosis, investigators suspected that the defect in this disorder might involve the escape of cystine from the lysosome to the cytosol.

3.2.1.5 Lysosomal cystine transport studies

The concept of a lysosomal cystine transport defect was difficult to test experimentally. In 1979, Reeves solved the problem by introducing a technique using methyl esters of amino acids to load the lysosomes, and the rate of egress of labelled cystine could then be monitored. These methyl esters are rapidly taken up into the lysosomes, and are hydrolyzed by the acid hydrolases yielding methanol and the ionized form of the corresponding amino acid.

In case of cystinotic cells, cystine dimethyl ester was found to be a suitable substrate. Two approaches were studied: firstly the use of (^{35}S)cystine dimethyl ester for loading and high voltage electrophoresis for (^{35}S)cystine determination. It was found that (^{35}S)cystine was cleared much slower from cystinotic cells ($t_{1/2}$= 80 to over 500 minutes) than from normal cells ($t_{1/2}$= 42.7± 3.1 minutes). One of the major drawbacks was the possibility of dilution of the specific radioactivity of (^{35}S)cystine by the large endogenous cystine pool of the cystinotic but not the normal cells. This problem was overcome by using a second approach: non radioactive cystine dimethylester for loading and subsequent measurement of non radioactive cystine by the cystine binding protein assay. In 1982, Steinherz et al applied the latter technique and showed that leucocytes from two normal individuals lost half their total cystine in 27 and 67 minutes respectively compared to 366 minutes and infinity in case of leucocytes from two cystinotic patients.

These radioactive and non radioactive experiments were performed on granular

fractions of leucocytes (Gahl et al, 1982a;b). The results were similar to those of whole cell experiments suggesting clearly that there is a defective cystine transport across the lysosomal membrane. It is worth noting that in those cystinotic granular fractions methionine and tryptophan were removed with normal half times suggesting that in cystinosis the defect did not represent a generalized abnormal lysosomal amino acid transport.

3.2.1.6 Lysosomal cystine transport: carrier mediated

The mode of transport of cystine was defined as carrier mediated; this was verified by the demonstration of countertransport and transstimulation. Trans-stimulation, is demonstrated experimentally when tracer amounts of radiolabelled material will cross a membrane at a high rate if there is a considerable concentration of its non radioactive form on the opposite side of the membrane. Therefore if non radioactive cystine loaded lysosomes take up (^{3}H)cystine more rapidly than lysosomes not loaded with cystine this suggests that a carrier for cystine must be present in the lysosomal membrane. It was found that only extralysosomal non radioactive L-cystine but not D-cystine competed with (^{3}H)cystine for uptake into these cystine loaded normal granular fractions (Gahl et al, 1983) establishing the stereospecificity of the carrier for the "L" isomer. In contrast, cystinotic granular fractions took up virtually no (^{3}H)cystine (Gahl et al, 1983). It was also shown that leucocyte granular fractions (^{3}H)cystine uptake was proportional to the level of non radioactive cystine, loading up to 3 nmol 1/2 cystine / unit β-hexosaminidase above which uptake plateaued indicating saturation kinetics.

The cystine carrier was described as being specific for compounds of chain length 6 sulfur or methylene units having an amine group at each end. Sulfur compounds resembling cystine such as cystathione and cysteamine compete for countertransport as they are recognized by the cystine carrier.

Structural specificity and stereospecificity, together with temperature dependence, countertransport and saturation kinetics confirmed that cystine transport across the lysosomal membrane is carrier mediated. The virtual absence of such a transport system in cystinotic patients resulted in defective egress of cystine across the lysosomal membrane (Gahl et al, 1982 a,b; Jonas et al, 1982).

3.2.1.7 Methods of measuring cystine content

Cystine content can be measured in several ways. One of the earliest procedures consisted of the reduction of cystine to cyst(e)ine and its qualitative measurement by the cyanide nitroprusside reaction (Brand et al, 1930). More recently, routine quantitation of

cystine was performed by automated amino acid analyzers (Schneider et al, 1967a; Lee Ply, 1974). Other cystine assays used were ion exchange paper (Schneider et al, 1968), and thin layer chromatography (States and Segal, 1969). One of the most commonly used techniques is the sensitive competitive protein binding assay using an Escherichia Coli cystine binding protein (Oshima et al, 1974).

3.2.1.8 Factors affecting the recovery of cystine from cystinotic cells

Factors such as storage and incubation temperatures of cystinotic fibroblasts, method of fibroblast detachment , cell passage number, cystine, albumin and chloroquine content of the culture medium, and pH of the medium were found to affect the recovery of free cystine from cystinotic cells

It was shown that the recovery of free cystine from cultured skin fibroblasts declined about 50% after 24 hours of storage at the following temperatures -20°C, -70°C, and -196°C and about 25% more following another six days at those temperatures; the decline was slightly greater at -20°C than the other temperatures (Kroll et al, 1974). Increased incubation temperature of cystinotic fibroblasts to 40°C or 43°C produced a 70%-80% decrease in lysosomal cystine content within 24 to 48 hours (Lemons et al, 1984).

The method of fibroblast detachment was found to affect the the recovery of amino acids in general and to a lesser extent cystine. It was demonstrated that scraping the fibroblasts with a "rubber policeman" decreased the recovery of amino acids compared with detaching them by trypsinization (Kroll et al, 1974). Crawhall et al (1977) showed that the length of time in cultivation affected the content of cystine in cystinotic fibroblasts. They found that the cystine content increased with increased cell passage number. The effect of the composition of the culture medium was emphasized in two studies by Thoene and Lemons, 1980;1982. They demonstrated that the cystine content of cystinotic fibroblasts varied with the addition of bovine serum albumin and cystine to the medium; and when placed in growth medium containing chloroquine, cystinotic cells were found to lose their stored cystine: 50% or more disappearing in 2 hours and 80% in 4 hours (States et al, 1983). Chloroquine is a potent lysosomotropic drug which accumulates in lysosomes, raises the lysosomal pH, and inhibits the proteolytic activity of cathepsin B_1 and other lysosomal enzymes. Thoene et al (1977) showed that chloroquine acts as a lysosomal inhibitor of protein catabolism and that reaccumulation of cystine by cystine-depleted cystinotic cells was prevented when the cells were incubated in cystine free medium containing chloroquine. It was also suggested that the increase in intralysosomal pH caused by chloroquine, could affect the solubility of cystine contributing to its marked loss.

The free cystine content of human cystinotic fibroblasts was found to vary with the pH of the culture medium (Ritchie et al, 1981). It was found to be highest at alkaline pH and lowest at acidic pH. When the cells were placed in culture medium at an acid pH (pH 6.3), they lost 50% of their free cystine and >95% over a period of 30 hours.

3.2.1.9 Therapeutic value of of administrating cysteamine in cystinosis

From the initial observation that cysteamine depletes cystine from lysosomes, it was believed at first, that it enters into a sulfhydryl-disulfide exchange reaction with the accumulated lysosomal cystine, generating amino acid molecules small enough to escape via hypothetical pores in that organelle. However, it has since been shown that removal of cystine actually occurs instead in a modified form by the transport system for basic amino acids. The mixed disulfide generated by the sulfhydryl-disulfide exchange, being itself a cationic amino acid, serves as a substrate for the system characterized for transporting basic amino acids. Exodus of ^{14}C-Lysine was shown to be stimulated by the external presence of the mixed disulfide of cysteine and cysteamine (Christensen, 1988). Thus the therapeutic utility of cysteamine acquired a new explanation through its generation of a specifically transportable derivative of cystine.

The follow up of 93 children treated with cysteamine showed that the mean leucocyte depletion was 82% (Thoene et al, 1976). In addition significant improvement of growth was noted during the first year of treatment and every year after. Cysteamine has recently been shown to prevent the development of Fanconi syndrome in a patient treated from 5 weeks of age (Da Silva et al, 1985) but not in one treated from 9 weeks of age (Gradus et al, 1986). Corneal crystal formation can be reversible after using cysteamine eye drops (Kaiser-Kupfer et al, 1987). In utero therapy with cysteamine seems reasonable as cystine accumulation begins early in fetal life, but the risks of teratogenic effects of the free thiol appear prohibitive at this time (Gahl et al, 1989).

3.2.2 Sialic Acid Storage Disorders

The free sialic acid storage disorders include Salla disease and the more severe infantile free sialic acid storage disease (ISSD). These disorders show autosomal recessive inheritance. Patients store excessive amounts of N-acetylneuraminic acid (sialic acid) in their tissues due to impaired free sialic acid transport across the lysosomal membrane. Most of the affected patients were originally from an area in northern Finland called Salla, from whence comes the name "Salla disease". Patients with Salla disease present at 3 - 12 months of age and show psychomotor delay, ataxia and moderate to severe impairment of intelligence, with slightly reduced life span. Patients with ISSD have a more severe clinical

picture and die in the first few years of life. They present at birth with hepatosplenomegaly, dysostosis multiplex, coarse facial features, and severe mental and motor retardation (Ghal et al, 1989).

The intralysosomal localization of sialic acid in Salla disease was first demonstrated by histochemical methods (Virtanen et al,1980). Differential centrifugation of ISSD fibroblasts has also shown that the accumulation of free sialic acid is mainly in the lysosomal-rich granular fraction (Hancock et al, 1980). These findings were further confirmed by electron microscopy which showed enlarged lysosomes containing fibrillogranular material forming multilinear arrays or lamellar stacks (Wolburg-Buchholz et al, 1985).

The underlying defect in Salla disease and ISSD was suggested to be defective transport of sialic acid across the lysosomal membrane based on two observations: 1) the major enzymes involved in the metabolism of sialic acid are normal, 2) the existence of a cytoplasmic but not a lysosomal enzyme for the catabolism of free sialic acid.

Lysosomal transport studies were carried by loading normal fibroblasts with N-acetylmannosamine (ManNac), a precursor of sialic acid (Thomas et al, 1985), and absolute amounts of free sialic acid were measured by high performance liquid chromatography (Silver et al, 1981). Lysosomal-rich granular fractions were prepared by Percoll gradient centrifugation and ManNac-derived sialic acid was found to fractionate with the lysosomal marker enzyme. It was shown that free sialic acid lost from inside lysosome-rich fractions was recovered outside, and that the velocity of this reaction was linear with time through 15 minutes. Failure to demonstrate saturation kinetics was possibly due to inability to load normal lysosomes sufficiently. In Salla disease granular fractions did not exhibit a similar loss over the same period of time. The egress of sialic acid from normal lysosome-rich granular was temperature dependent suggesting the presence of a carrier mediated transport process (Tietze et al unpublished observations as cited in Gahl et al, 1989). When Salla disease, ISSD, and control normal fibroblasts were exposed to (^{3}H)sialic methyl ester, the radioactivity appeared in the lysosomes as free sialic acid. The follow up of this radioactivity demonstrated a prolonged $t_{1/2}$ of sialic acid egress from the mutant lysosomes compared with normal (Mancini et al, 1986).

3.2.3 An inborn error of vitamin B_{12} metabolism: cblF

In general, cobalamins (Cbls) consist of a corrin ring, a central cobalt atom, and various axial ligands. Ten different inherited defects, defined by complementation analysis, are known to impair the pathways of Cbl metabolism in humans (Fenton and Rosenberg, 1989). CblF is a newly described mutation, where the defect appears to be in the transport

mechanism by which Cbl is released from lysosomes. As a result there is impaired synthesis of both methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) and accordingly deficient activity of both methylmalonyl-CoA mutase and N^5-methyltetrahydrofolate:homocysteine methyltransferase *(Fig 1.5)*. One of the two described cblF patients had both methylmalonic aciduria and homocystinuria, while the other only had mild methylmalonic aciduria. Normal serum cobalamin and transcobalamin II concentrations in these patients indicated that the defect is mainly in the release of vitamin B_{12} from lysosomes. The mode of inheritance of the cblF disorder is not yet established due to the paucity of the number of patients.

Cultured fibroblasts from one of the reported cblF patients were found to accumulate unmetabolized, non protein-bound cyanocolabamin (CN-Cbl), suggesting that these cells are deficient in the mediated process by which cobalamin exits from lysosomes. Genetic complementation analysis between the cblF patient fibroblasts and fibroblasts whose complementation groups have been previously determined allows the cblF group to be distinguished from other defects (Rosenblatt et al, 1985; 1986; Watkins and Rosenblatt, 1986). Recently, the EM radioautography of cblF fibroblasts provided morphological evidence that accumulation of vitamin B_{12} occurs within the electron dense lysosomes. When control and cblF fibroblasts were incubated in a medium containing (^{57}Co)-CN-B_{12} and 21% human serum as a source of transcobalamin II, it was found that cblF cells accumulated 4-8 times more radiolabelled cobalamin than control cells. The control and the cblF fibroblasts were then processed for electron microscopy and the radioautographic reaction was quantitated. The findings showed that in the cblF cells 65% of the radiolabel was associated with lysosomes and 8% was associated with electron lucent endosomes. In contrast , in the normal cells 56% of the label was found within the cytoplasm and only 5% was associated with the lysosomes (Vassiliadis et al, 1988).

4. Cystine storage in I-cell disease

I-cell disease and cystinotic fibroblasts were selected for this study as they have been reported to show intralysosomal accumulation of cystine (Tietze and Butler, 1979). I-cell disease (ML II) is an autosomal recessive disease manifested in early infancy by stunted growth, profound psychomotor disturbance, widespread skeletal deformities and death within the first decade (Leroy and Martin, 1975). I-cell fibroblasts and other cells demonstrate a deficiency or a virtual absence of several lysosomal hydrolases. In 1972, Leroy et al performed biochemical studies in six patients with I-cell disease and showed that in their fibroblasts, the activities of several lysosomal enzymes were as follows: β-galactosidase 2% of normal, β-glucosaminidase 8% of normal, β-glucuronidase 7% of

normal, α-galactosidase 10% of normal, and arylsulfatase A 5% of normal, whereas both β-glucosidase and acid phosphatase were not deficient. The deficient enzymes resulted in intralysosomal accumulation of partially hydrolysed molecules which appeared as phase-dense inclusions in the fibroblasts of affected individuals. As a result the cells were termed "Inclusion cells", and subsequently the disease was termed "I-cell disease".

Figure 1.5 **Reactions catalyzed by cobalamin coenzymes in mamalian tissues**

FIGURE OMITTED DUE TO COPYRIGHT RESTRICTIONS

source: Figure 82-2, p2067. Fenton, Rosenberg: Inherited disorders of cobalamin transport and metabolism. In: The Metabolic Basis of Inherited Diseases. Scriver, Beaudet, Sly, Valle (eds), McGraw Hill, New York, 1989.

The primary biochemical defect in I-cell disease was found to be in the packaging process of the lysosomal enzymes required for their proper targeting to the lysosomes (Hickman and Neufeld, 1972; Hasilik and Neufeld, 1980; Hasilik et al, 1981; Reitman et al, 1981). In normal cells, targeting of the lysosomal hydrolases to the lysosomes is mediated by receptors that bind a Mannose-6-phosphate marker on the enzymes. In the cis Golgi, this recognition marker is generated through the sequential actions of two enzymes; a phosphotransferase and a phosphodiesterase. In I-cell disease N-acetylglucosaminyl-1-phosphotransferase, the enzyme which catalyses the first step, is defective (Nolan and Sly, 1989).

Other biochemical defects have been observed in I-cells. In 1979, Tietze and Butler, found in seven out of ten I-cell disease fibroblasts abnormally high levels of free cystine comparable to those seen in homozygous cystinotic cells. They also demonstrated that the increased cystine content seen in I-cells was unique to cystine and did not reflect a generalized increase in the total free amino acid content of these cells. Jonas et al (1981) showed that in I-cell disease, cystine accumulates in lysosomes similar to what is seen in cystinosis.

The intralysosomal pH in normal, cystinotic , and I-cell fibroblasts were compared (Oude Elferink, Harms and Tager as cited in Kooistra et al, 1984). There was no difference found in the intralysosomal pH between the cystinotic and normal cells ; but in I-cell disease the pH was lower than normal. The significance of these results was not clear. Other theories to explain of the increased cystine in I-cell fibroblasts were suggested: a) abnormal lysosomal membrane integrity in I-cell fibroblasts (i.e. defective lysosomal enzymes required for the integrity of the membrane, abnormal charged groups at the binding site, change in pH and potential gradient), b) blockage of cystine egress by the presence of undigested inclusions (Greene et al, 1985)

More recently, in 1986, Tietze et al suggested, based on (^{35}Scystine) egress studies, that I-cell fibroblasts, like cystinotic cells possess a defective carrier mechanism for cystine transport. They measured the rates of clearance of free (^{35}S cystine) in normal, cystinotic and I-cell intact fibroblasts. They found that the loss of radioactivity occured slowly in the two mutant cell lines compared to the rapid loss from normal cells. They also reported decreased rates of efflux of free cystine from lysosome-rich granular fractions of cystinotic and I-cell fibroblasts. They proposed that this could be due to either defective packaging of cystine carrier glycoprotein lacking the Man-6-P marker, or due to inadequate proteolytic processing of an inactive precursor form of the carrier as a result of low activity of lysosomal proteinases in I-cell fibroblasts.

Another secondary defect in I-cell disease fibroblasts is the high content of sialic acid-containing residues due to the lack of neuraminidase activity. It was suggested that this defect might change the membrane potential (Greene et al, 1985). This altered membrane structure has also been found to confer increased sensitivity to freezing (Sly et al, 1976).

5. Review of the methodology

"The general problems encountered when one tries to isolate subcellular organelles in an undamaged state are mainly : how to break cells without breaking the organelle of interest, how to maintain the organelles intact as they are removed from their normal cellular milieu, and finally how to separate the organelles from each other" (Dean, 1977).

5.1 Cell Harvesting

Two main methods of harvesting fibroblast cell lines were mostly used;

1. Controlled trypsinization (Rome et al, 1979; Harris,1981; Thomas et al, 1985; Renlund et al, 1986a).
2. Scraping with a rubber policeman (Harms et al, 1980; Gieselmann et al, 1983; Mokrasch, 1985; Renlund et al, 1986b)

It has been reported that the method of fibroblast detachment affects the recovery of amino acids in general and to a lesser extent cystine e.g. the recovery of amino acids was decreased when fibroblasts were scraped with a rubber policeman compared with their detachment by trypsinization (Kroll et al, 1974)

5.2 Cell disruption

Several methods of cell lysis have been described in the literature depending on the the type of cells and on the complexity of of their intracellular and extracellular matrices.

I- Homogenization:

Homogenization is the most popular method of cell lysis, however, it has been applied in many different ways according to several variables i.e.

i- Type of pestle used being either tight or loose fitting.

ii- Lysis buffer being hypo-osmotic or iso-osmotic.

iii- Number of strokes.

iv- Type of homogenizer, glass (Gieselmann et al, 1983) or metal (ball bearing homogenizer) (Balch and Rothman, 1985).

The interaction of the above mentioned variables can be examplified in the following techniques:

1- Uniform lysis of cells exposed to hypo-osmotic conditions was done by the use of a loose fitting pestle with 2 to 5 strokes (Mokrasch, 1985).

2- Fibroblasts suspended in isotonic sucrose were broken with 20 strokes by a tight fitting pestle (Gieselmann et al, 1983).

II- Nitrogen cavitation

Cells suspended in 0.25 mol/L sucrose were disrupted by nitrogen cavitation [30 psi (2.0 atm), 10 minutes] (Renlund et al, 1986a).

III- Sonication

Sonication, as a means of cell lysis, has been applied for three 10 second periods (Thomas et al, 1985); however, in general sonication is considered as an inconsistent method of cell lysis.

IV- Finally, cell disruption was done by passing the suspension through the tip of a pipette i.e. drawing and expelling the cell suspension 15 times through a 10 mL glass pipette (internal diameter of the tip 1.45 mm) (Harms et al, 1980; Pisoni et al, 1985; 1987), whereas, Renlund et al (1986b) lysed the cells by passing the cell suspension 50 times through the tip of a 1 mL Eppendorf pipette.

5.3 Methods of isolation of lysosomes

Centrifugal techniques depends mainly on differences in size and / or density of the particles to be separated. Certain functional characteristics of lysosomes have been used to modify their behavior in centrifugation i.e. altering their density. In order to review the methods of the isolation of lysosomes, we classify them into two main categories:

5.3.1 with pre-treatment (alteration of lysosomal density)

5.3.2.without pre-treatment (no alteration of lysosomal density)

5.3.1 Isolation of lysosomes with pre-treatment (alteration of lysosomal density)

The sedimentation coefficients of lysosomes, mitochondria, and peroxisomes overlap, especially since the lysosomal population is extremely heterogenous, with only one common characteristic, the content of acid hydrolytic enzymes. Therefore, pre-treatment of lysosomes has been used in attempts to allow better separation.

a) Triton loading technique

When injected in experimental animals, Triton WR-1399 was deposited in secondary lysosomes and induced a decreased equilibrium density of these organelles causing them to float and so could be collected from the interphase between the 14% and 35% sucrose layers of the gradient (Wattiaux et al, 1963; Leighton et al, 1968). This technique has proven to be effective in purifying lysosomes from many organs other than liver, such as lymphoid tissue, spleen or kidney or in getting other constituents such

peroxisomes and mitochondria free of lysosomes (Dean, 1977). The drawbacks of this method were found to be mainly the swelling of lysosomes and the interference with lipid metabolism (Huterer et al, 1975; Dean, 1977).

b) Iron loading technique

This method consisted of repetitive injections of an iron-sorbitol-citric acid complex which usually increases ferritin synthesis (Arborgh et al, 1974; Glaumann et al, 1975; Hultcrantz and Glaumann, 1982); and since ferritin is metabolized in the lysosomes, it accumulates together with haemosiderin in the lysosomal compartment resulting in a marked increase in the density of the lysosomes. The loaded lysosomes can then be isolated by both differential and isopycnic centrifugation in a continuous sucrose gradient, however, the main drawback lay in the fact that the ferritin loaded lysosomes may have altered functional attributes.

Other techniques such as injection of colloidal gold (Henning and Plattner, 1975) or administration of dextran (Thines Sempoux, 1973) have been described but did not gain general use. In both methods the introduced compounds accumulated in the lysosomes, increasing their densities and allowing their separation from the mitochondria and the peroxisomes.

5.3.2 Isolation of lysosomes without pre-treatment (no alteration of lysosomal density)

a) In 1978, Wattiaux et al described a method for the isolation of rat liver lysosomes without any alteration of their density. The technique consisted of centrifugation in a discontinuous metrizamide gradient. The two encountered drawbacks were the high cost of metrizamide and the occasional aggregation of lysosomes and other organelles (Glaumann et al, 1982).

b) Another technique was described by Yamada et al, in 1984, where lysosomes were isolated from livers of untreated rats. The post-nuclear supernatant was incubated with 1 mmol/L Ca^{2+} which causes swelling of mitochondria and alteration of their isopycnic density. Lysosomes were then separated from the swollen mitochondria by Percoll density gradient centrifugation followed by repetitive washing procedures.

c) Isolation of lysosomes by free flow electrophoresis was described by Harms et al, 1980.

d) Lysosomes from human skin fibroblasts were isolated by differential pelleting followed by Percoll gradient centrifugation and finally repetitive washing procedures (Pisoni et al, 1985).

5.4 Protein determination

Lowry's method is the most widely used protein assay; however, it was found that Percoll causes a background color with the Folin-Ciocalteau reagent, interfering with the procedure (Bensadoun et al, 1976; Rome et al, 1979). Modifications were attempted using Percoll solutions for the preparation of the blank. However, since a Percoll gradient is being analyzed, the accurate concentration of the Percoll in the samples is not known and it is not practical to run a separate control for each sample especially when a large number of samples have to be assayed (Khan et al, 1981). Several methods have been described to deal with interfering substances, for instance, removal of the precipitate formed in the assay by centrifugation (Raja and Klein, 1975) or precipitation of protein before assay (Bensadoun, et al, 1976).

In 1979, Terland et al described another technique recommending the use of the Coomassie blue method of Bradford (1976). However, this procedure did not give satisfactory results due to the development of intense turbidity in the assay tube in the presence of Percoll (Khan et al, 1981).

A method for quantitation of protein in the presence of Percoll (maximum concentration 30%) was developed by khan et al (1981) using a dual wavelength correction. This method was based on monitoring the absorbance at 750 and 420 nm of color developed by protein plus Percoll with Folin Ciocalteau reagent. The difference in the extent of absorbances was used to determine the protein concentration in samples containing protein plus Percoll. Standard curves for Percoll and protein in 0.25 mol/L sucrose were linear.

5.5 Amino acid Analysis

Interest in the determination of free amino acid content in biological fluids is as old as the knowledge of amino acids (Berzelius, 1833; Frerichs, 1854; Fischer and Bergell, 1902 as cited in Deyl et al 1986). Column liquid chromatography was rediscovered in 1941, by Martin and Singe, who were aiming at the separation of amino acids. In 1944, Consden et al, improved the method by introducing paper chromatography of amino acids, and it was soon applied to the analysis of free amino acids in blood and urine (Dent, 1947). Automated sample collectors were introduced into column liquid chromatography in 1950, making the separation easier. The first fully automated amino acid analyzer was constructed by Spackman et al, 1958 (as cited in Deyl et al, 1986). Paper chromatography was slowly overtaken by thin layer chromatography and ion exchangers dominated the area of amino acid analysis in the 1960s and 1970s.

Prolifing of amino acids is of great importance today and has found broad application in clinical practice. The main diagnostic applications of free amino acid profiling have been to urine (Jagenburg, 1959), capillary and venous blood (Emery et al, 1970), serum, plasma (Armstrong and Stave, 1973), and amniotic fluid (Bremer et al, 1981 as cited in Deyl et al, 1986). Amino acid analysis was also performed on other biological fluids such as saliva (Moor and Gilligan, 1951), tears (Fleischmayer and Wiechert, 1963), synovial fluid, cerebrospinal fluid, exudates and transudates (Biserte et al, 1963), feaces (Levy et al, 1969), sweat (Liappis and Hungerland, 1972), sperm and breast milk (Brown-Woodman and White, 1974; Hyanek et al, 1968 as cited in Deyl et al, 1986). In the case of tissues and cell populations, free amino acids were determined in fibroblasts cultures (Holden, 1962), hair (Pollit et al, 1968), nails , teeth, bones, muscles (Curtius et al 1968), red blood cells (Levy and Barkin, 1971), homogenates of liver cells and brain cells (Perry et al, 1971), and leucocytes (Houpert et al, 1976).

5.5.1 Preparation of biological samples for amino acid analysis

Deproteinization of biological samples is an essential step in their preparation for amino acid analysis as the presence of protein has a considerable influence on the determination of free amino acids. Proteins may adhere to ion exchangers in columns causing peak spreading and resulting in an increased column back pressure. Other special sample treatment might need to be done before analysis; for instance desalting in case of high concentrations of salts particularly seen in urine samples (Jagenberg, 1959), extraction of lipidic components as in the case of hyperlipaemic serum or breast milk (Peters et al, 1969), removal of excessive amounts of ammonia in urine samples (Turnell and Cooper, 1984).

Deproteinization can be carried out either by chemical reagents or by physical techniques, and can result in some losses due to the adsorption of amino acids on the protein surface. These losses are particularly important when the amino acids are present in small amounts such as in the case of aspartic acid, asparagine, citrulline, ornithine, cystine and tryptophan. Precipitation of proteins by chemical reagents is the oldest deproteinization technique (Moore and stein, 1948). Sulfosalicylic acid was found to be the most suitable deproteinization reagent. It has been used as a 20% solution (Moore and Stein, 1948), or in the solid form, especially when working with small protein content in the samples. In the latter case, it was suggested to use as little of the precipitating reagent as possible i.e. 20-50 mg sulfosalicylic acid / mL plasma (Mondino, 1970). However, in case of high protein content in the sample as in fibroblast cultures, it was recommended to apply a concentration of sulfosalicylic acid as high as 200 mg / mL (Holden, 1962). Perchloric, trichloroacetic

and picric acids were also used as deproteinizing reagents especially for tissue samples. Another chemical technique of deproteinization was performed using ion exchangers e.g. a cation exchanger in the H^+ form such as chromobead (A). Amino acids are bound to the ion exchanger by shaking the solution with the ion exchange resin. The mixture is then centrifuged, the supernatant containing the proteins is discarded and the ion exchanger bound amino acids are released with 0.1mol/L HCl (Deyl et al, 1986).

Ultrafiltration seems to be the most popular.physical method of deproteinization. Amicon centriflo membrane cones of cF25 - cF50 (molecular weight cut off 25,000 and 50,000 respectively) were used and were found to remove 99.8 ± 0.06% and 99.5 ± 0.31% (mean ± S.D) of plasma protein (Blanchard, 1981). Ultracentrifugation is another physical means used in separating protein from free amino acids. However, it is cumbersome (Cohen and Strydom, 1988) and it is hence, rarely used. For the sake of completeness, a third physical method of deproteinization should be mentioned, that is deproteinization using thermal fixation on cellulose fibres, where the protein fraction is immobilized on cellulose fibres by increased temperature, acetic acid vapour or by drying (Guthrie and Susi, 1963).

5.5.2 Chromatographic separation and profiling of free amino acids

In principle, separation of amino acids is based either on the charge or on hydrophobicity differences and in liquid column chromatography, the type of chromatography used depends on whether one is separating derivatized or underivatized amino acids. Therefore, two possibilities could be applied: Firstly, underivatized amino acids can be separated by cation exchange chromatography followed by a post-column derivatization reaction for detection (it is important to note that with the exception of the aromatic amino acids and few others, most underivatized amino acids cannot be detected by UV absorption unless if very short wave length is used). Secondly, amino acids can be converted into suitable derivatives before separation and then the separation is run in the reversed-phase mode. None of these available chromatographic techniques fit all demands because of the many possible applications of amino acid analysis, and each technique exhibits specific advantages and disadvantages.

5.5.2.1 Post-column derivatization

Usually, separations performed with post-column derivatization are carried out on cation exchangers with a gradient of acidic buffers. The more acidic amino acids (possessing an additional carboxyl group) elute first and those with more than one primary amino group or possessing a guanidyl residue elute at the end of the chromatogram. Then,

the separated amino acids are converted into coloured derivatives e.g. ninhydrin derivatives for spectrophotometric detection, or into fluorescent derivatives e.g. O-phthalaldehyde (OPA) derivatives for fluorescence detection.

Ion exchange chromatography of underivatized amino acids with ninhydrin detection has been the standard method because of the number of compounds separated and detected within the profile i.e. 52 ninhydrin-positive compounds can be separated. Replacement of ninhydrin by fluorescamine (Udenfriend et al, 1972; Stein et al, 1973) promised to enhance the sensitivity of all amino acid analysis, except those with secondary amino groups such as proline and hydroxyproline. Analysis at low pico mole levels were possible (Stein and Udenfriend, 1984). However, the use of another fluorogenic reagent, O-phthalaldehyde (OPA) has superceded the use of fluorescamine, in most laboratories (Benson and Hare, 1979). Post-column derivatization with OPA therefore offers greater sensitivity than the classical ninhydrin procedure. Although OPA does not react with secondary amino acids, it is possible to generate a primary amine from secondary amines by oxidation with sodium hypochlorite (Bohlen and Mellet, 1979). It was shown that the potential limits of detection with OPA are below 100 pmol (Cooper et al, 1984); however, in practice this limit seemed to be at 50-100 pmol for proline, and 5-10 pmol for the other amino acids (Dong and Gant, 1985).

For completeness, two more post column fluorescent reagents should be mentioned. Firstly, a technique, using 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole (Watanaabe and Imai, 1983 as cited in Deyl et al,1986) was described. It demonstrated sensitivity in the picomole range. It was also tested for clinical applicability and was proven to be suitable for the analysis of free amino acids in blood discs. Secondly, a reagent known as Pentane-2,4-dione and formaldehyde has been suggested for amino acid analysis by Kakehi et al, 1985; however, it was found to be much less sensitive than OPA and the color yields of proline and tyrosine were very low.

One often encountered problem in the amino acid profiling of tissues with ion exchange chromatography is the occurrence of one to three fast peaks at the start of the chromatogram. This fraction has been called the "Taurine fraction", representing a complex mixture of more than ten components of which taurine, phosphoserine, phospho ethanolamine, glycerophospho-ethanolamine, hypotaurine, cysteic acid and cysteine sulphinic acid have been characterized (Deyl et al, 1986). These individual highly acidic and highly ionized compounds have very close retention times and ion exchange chromatography fails to separate them adequately under the conditions used for routine amino acid profiling.

5.5.2.2 Pre-column derivatization

Reversed phase chromatography is the preferred separation procedure if the aim is to separate amino acid derivatives. Octadecysilane hydrophobized sorbents have been widely used. A variety of solvent systems are available, mostly containing methanol and acetonitrile as the organic component of the mobile phase (Deyl et al, 1986). Numerous compounds have been used in pre-column derivatization of amino acids, mostly electrophiles capable of reacting with the free α-amino group. The most popular is phenylisothiocyanate (PITC), known as the Edman reagent, which forms phenylthiocarbamyl derivatives of amino acids (Heinrikson and Meredith, 1984). There are several key features of the phenylthiocarbamylation chemistry that encouraged the development of PTC amino acid analysis: a) specificity of PITC has been well characterized with no evidence for the formation of any disubstituted derivatives with tyrosine or histidine, b) reagent interference is minimal, c) PTC derivatives have been detected at the 1 pmol level (Cohen and Strydom, 1988)

Other reagents include 5-dimethyl aminonaphthalene-1-sulfonyl (dansyl) chloride, and its analog 4-dimethylamino-azobenzene-4-sulfonyl (dabsyl) chloride, 2,4 dinitrofluorobenzene, and 4-chloro-7-nitrobenzo-2-oxo-1,3-diazole (NBD-Cl) (Cohen and Strydom, 1988). HPLC analysis of Dansyl amino acid derivatives was judged to be moderate in terms of separation, sensitivity, precision, and time of analysis. However, various unidentified and interfering peaks were often encountered, and it was unknown whether both mono and didansyl derivatives were being formed or whether reaction products were using the extraneous peaks. It was suggested that the use of dansyl derivatives might not be appropriate in various biological applications (Wilkinson, 1978). On the other hand , although the Dabsyl derivatization procedure was found to be simple and rapid, it presented a major drawback : the formation of multiple derivatives with bis-amino acids which eluted from the column at the same time as other monoderivatives.

O-phthalaldehyde (OPA) has been also used in pre-column derivatization of amino acids. In conjunction with a thiol reagent, OPA reacts with primary amino groups to form highly fluorescent isoindole products (Hill et al, 1979). The wide use of OPA in pre-column systems is due to several advantages such as the high fluorescent yield exhibited by its derivatives, the solubility and stability of the derivatives in aqueous buffer, the rapidity of the reaction, and the absence of OPA interference with the reaction. However, OPA has exhibited some major drawbacks, namely the inability to form fluorescent derivatives with secondary amino acids, the formation of double peaks with histidine, lysine, and ornithine; and lastly the instability of the derivatives (Mc Clung and Frankenberger jr, 1988).

A reagent originally introduced as a blocking group in peptide synthesis, FMOC-Cl (9-fluorenyl-methyl Chloroformate), has been also used for amino acid analysis (Einarsson et al, 1983). FMOC-Cl was found to react rapidly with both primary and secondary amino acids forming highly fluorescent derivatives. However, disadvantages, such as the formation of mono or disubstituted derivatives with histidine, and reagent interference, were encountered.

Derivatization of the carboxyl group of amino acids was tried (Yoshida et al, 1985), but did not gain popularity. Phenylthiohydantin (PTH) derivatives were also used and found to offer a rapid HPLC analysis, fairly good resolution, sensitivity, precision and derivative stability. However, the lengthy process of forming PTH derivatives made this amino acid derivatization technique undesirable (McClung and Frankenberger jr, 1988).

6. Objective

The purpose of this study is to develop a method for the measurement of the whole lysosomal amino acid profile in normal fibroblasts, and to test it in cystinotic and I-cell disease fibroblasts where specific abnormalities are expected. Finally, if successful the methodology could be used to screen other candidate genetic diseases.

7. Hypotheses

1- Intralysosomal concentrations of amino acids would be increased if a lysosomal transport defect were present.

2- If the cystine carrier transported amino acids other than cystine i.e. not completely specific, concentrations of these other amino acids would be increased in cystinosis.

3-The cystine transporter may be deficient in I-cell disease lysosomes due to defective targeting. If there are other amino acid transporters in the lysosomes, these may also be affected and increased intralysosomal concentrations of the corresponding amino acids could be observed.

4- The features one might expect in disorders of lysosomal transport are abnormal lysosomal morphology, or abnormal storage of material (particularly localized to lysosomes), and deficiencies of vitamins, minerals, or other essential compounds. Some possibilities could include Menke's disease (copper storage), Chediak Higashi syndrome (azurophilic granules accumulation), Indian childhood cirrhosis (iron storage), and ceroid lipofuscinosis (lipofuscin storage).

Chapter two: Methodology

1- Human Fibroblast Culture

Fibroblast cultures were either started from cell lines purchased from the Human Genetic Mutant Cell Repository (Camden, New Jersey) or from human skin punch biopsies.

1.1 Reagents

1) Minimum Essential Medium (MEM, Gibco Cat. # 410-1100)

Dissolve one package of MEM in 1000 mL deionized water. Add 2 g $NaHCO_3$ and mix until dissolved. Add 10 mL Pen / Gent. Mix well, adjust the pH of the medium to 0.2-0.3 unit below the desired final working pH, as the pH units will usually rise 0.1-0.3 unit upon filtration. Filter under vacuum into a sterile flask through Nalgene 0.2 μ filter. Store at 4°C in two 500 mL sterile bottles.

2) MEM with 20% Fetal Calf Serum (FCS)

To 20 mL MEM (with Pen / Gent) add 5 mL fetal Calf Serum (FCS), 0.25 mL L-Glutamine, and 0.15 mL Fungizone. Mix well.

3) MEM with 10% Fetal Calf Serum (FCS)

To 90 mL MEM (with Pen / Gent), add 10 mL fetal Calf Serum (FCS), 1 mL L-Glutamine, and 0.6 mL Fungizone. Mix well.

4) Penicillin / Gentamycin (Pen / Gent)

Dissolve Penicillin G (Sodium salt , 5 x 10^6 IU), and Gentamycin sulfate (80 mg/mL) in 400 mL sterile deionized water, mix well and store in 10 mL aliquots at -20°C.

5) Fungizone (Gibco #600-5295) 20 mL

Reconstitute Amphotericin B (250 μg / mL) with 20 mL sterile deionized water, and store the vial at -20°C. It may be frozen and thawed several times without effect.

6) Fetal Calf Serum (FCS, heat inactivated, mycoplasma and viral tested, Gibco #230-6140).

Filter 100 mL through 0.22 μ corning filter membrane. Store in 10 mL aliquots in sterile tubes at -20°C.

7) L-Glutamine (200 mmol / L, Sigma G-3126)

Dissolve 2.92 g L-Glutamine in 100 mL deionized water. Filter through Nalgene 0.22 µ filter, and store in 5 mL volumes in sterile tubes at -20°C.

8) Trypsin (Gibco #610-5095) 2.5%

Reconstitute with 20 mL sterile deionized water. Store at -20°C. It may be frozen and thawed several times without effect.

Working Trypsin Solution:

Dilute one part in ten with Hanks Balanced Salt Solution. This solution may be stored frozen. When frozen and thawed more than once, it loses its effectiveness (a yellow or orange colour turns red when it loses its effectiveness).

9) Hanks Balanced Salt Solution (1 L, No Calcium, No Magnesium, Gibco #310-4170)

Store at 4°C once opened.

10) Dimethyl Sulfoxide (DMSO, 5 mL, Sigma D2650)

11) 10% DMSO / MEM

Add 1.0 mL DMSO to 9.0 mL MEM (with 20% FCS, P/G, FGZ, L-Gln). Mix well.

1.2 Procedures

Tissue culture procedures include: cell culture from skin biopsies, cell subculture, cell storage in liquid nitrogen, and thawing cells from liquid nitrogen (Kruse and Patterson, 1973).

1.2.1 Cell culture from skin biopsies

i- Collect a sterile skin biopsy 2 to 3 mm full thickness core of skin in a sterile tube containing MEM with 20% FCS. Start culture within the first five hours.

ii- Transfer biopsy core to a sterile 30 mm petri dish containing a large bead of MEM with 20% FCS.

iii- Using sterile scissors and forceps, cut the skin into very small fragments in a plane perpendicular to the surface.

iv- Add 1 mL MEM with 20% FCS to a sterile T-25 flask to cover the bottom surface of the flask.

v- Transfer the tissue fragments with a sterile pipet to the T-25 flask and carefully position the pieces in the flask to allow growth.
vi- Add 2 mL MEM with 20% FCS to the flask and let it sit at a tilted angle for several minutes to allow pieces to stick to the surface of the flask.
vii- Place the flask in the CO_2 incubator with the cap loose to allow exchange of air in the flask with the 5% CO_2 environment of the incubator.
viii- Examine under inverted microscope after 5 to 7 days without disturbing the fragments.
ix- Epidermal cells should begin to form after 5 days. Add 2 mL of MEM with 20% FCS and incubate further for several days.
x- If growth is progressing well draw off the medium and replace it with about 3 mL of MEM with 10% FCS. Continue the culture at 37°C, and check growth every 2 to 3 days under the microscope.
xi- Increase the amount of medium to about 6 mL during the following week.
xii- Subculture when there is a monolayer of confluent fibroblasts covering at least 50% or more of the flask surface.

1.2.2 Cell subculture[1]
i- Remove the medium from the flask and wash the cells with 3 mL Hanks Balanced Salt Solution.
ii- Prewarm 0.25% trypsin to 37°C and add 1 mL to the T-25 flask .The trypsin should just cover the cells. Incubate the cells at 37°C for 5-7 minutes until they detach from the surface of the flask. Tap and shake the flask to loosen the cells if necessary.
iii- Add rapidly, 3 mL of MEM with 10% FCS to stop the action of the trypsin. Wash the surface of the flask with the mixture of MEM and trypsin to remove any cells left attached to the surface.
iv- Transfer to a large T-75 flask or to 3 small T-25 flasks. Add 12 mL of MEM with 10% FCS to the large flask and 6 mL to each of the smaller flasks. Place the new cultures in the incubator with caps loosened.
v- Change most of the medium next day and replace with fresh MEM with 10% FCS.
vi- Incubate for 2 to 3 days, check under 10 X power for growth.
vii- Feed cultures by adding MEM with 10% FCS every 2 to 3 days.

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[1] Subculture was done 1 in 3 (T-75) flasks in case of normal fibroblasts and 1 in 2 (T-75) in case of mutant cell lines

1.2.3 Cell storage in liquid nitrogen

i- Proceed with the cell detachment as mentioned above and stop the trypsin action with 5 mL of 10% DMSO / MEM mixture.

ii- Transfer cells to small NUNC ampoules (1.8 mL maximum volume). Refrigerate for 3 hours at 4°C. Cells adjust to quiescence. Freeze at -20°C overnight, then transfer to -70°C for 12 hours and finally, store in liquid nitrogen.

1.2.4 Thawing cells from liquid nitrogen

i- Transfer the ampoule from liquid nitrogen immediately into a 37°C water bath until frozen liquid has just thawed.

ii- Dilute out DMSO by adding MEM with 20% FCS dropwise to a final volume of 6 mL.

iii- Transfer mixtures to a T-25 flask and incubate at 37°C and 5% CO2 overnight. Cells attach to the flask surface within several hours. Next day, change medium completely with fresh MEM with 20% FCS to remove DMSO.

iv- Carry on culture as usual, feeding with fresh MEM with 10% FCS.

2. Subcellular fractionation of human fibroblasts

Subcellular fractionation of the human fibroblasts is performed on a self generating Percoll gradient (Gieselmann et al, 1983).

2.1 Reagents

1) Percoll gradient

Percoll solution consists of silica particles coated with non dialysable polyvinyl pyrrolidone (PVP), with a density of 1.1130 ± 0.005 g/mL, and particle size of 15 to 30 nm in size and a mean diameter of 21 to 22 nm (Pharmacia Percoll manual, 1980).

When a solution of Percoll (in 0.25 mol/L sucrose) is centrifuged at > 10,000 x g, the coated silica particles will start to sediment, resulting in an uneven distribution of particles and thus forming a self generating density gradient.

The one step procedure for diluting Percoll

Percoll (from the bottle) may be diluted directly to make a final working solution of known density. The required volume of Percoll (from the bottle) is calculated using the following formula

$$V_0 = V\,(P - 0.1P_{10} - 0.9) / (P_0 - 1)$$

where,

V_0 = volume of Percoll (from the bottle) in mL

V = volume of the final working solution = 30 mL

P = desired density of the final solution = 1.065 g/mL

P_0 = density of Percoll from the bottle = 1.130

P_{10} = density of 2.5 mol/L sucrose[1] = 1.316 g/mL

Therefore, volume of Percoll required = $30\,(1.065 - 0.1)(1.316 - 0.9) / (1.130 - 1)$

$= 30\,(1.065 - 1.0316) / 0.13$

$= 30 \times 0.26 = 7.8$ mL

In order to prepare 30 mL of working solution of Percoll of density 1.065 g/mL in 2.5 mol/L sucrose, to 3 mL (1/10 of final desired volume) of 2.5 mol/L sucrose add 7.8 mL Percoll. Make up 30 mL with deionized water.

---

[1] The working solution of Percoll is made up with sucrose as the subcellular particles usually aggregate in the presence of salts (Pharmacia manual).

2) Density Marker Beads

Density determination is performed using "Density Marker Beads". These are derivatives of sephadex. There are 10 types, each type is color coded and modified to have a specific density in gradients of Percoll. These beads are used as an external marker and their densities (p= 1.037 - 1.138 g/mL) cover the buoyant densities of the vast majority of cells and organelles to be separated in Percoll. A sample of 25 µL of each colour bead (No 1-4 and 6-10) is layered on top of Percoll gradient (p= 1.065 g/mL). This tube is centrifuged simultaneously with the tube containing the biological samples.

3) Washing buffers

Buffer I: 10 mmol/L Na Phosphate, 0.14 mol/L NaCl, pH 7.4

Dissolve 0.69 g NaH_2PO_4 (monobasic, MW= 137.99) in 500 mL deionized water; solution A. Dissolve 0.71 g Na_2HPO_4 (dibasic, MW= 141.96) in 500 mL deionized water; solution B. To make up the buffer with pH 7.4, add 95 mL of solution A to 405 mL solution B. Dissolve 4.1 g NaCl in 500 mL phosphate buffer pH 7.4.

Buffer II: 0.25 mol/L sucrose, 1 mmol/L EDTA; pH 7.4

Dissolve 42.78 g sucrose (Ultrapure sucrose, MW= 342.30), and 0.186 g EDTA (MW= 372.2) in 450 mL deionized water. Adjust pH to 7.4 with 1N NaOH and qs with deionized water to 500 mL.

4) Isolation Buffer: 0.25 mol/L sucrose, 3 mmol/L imidazole HCl, pH 7.4

Dissolve 42.78 g sucrose and 0.16 g imidazole HCl (MW= 104.5) in 450 mL deionized water. Adjust pH to 7.4 with 1N NaOH and qs with deionized water to 500 mL.

2.2 Procedure

i- When cells are confluent (5 x 10^6 cells / flask) wash the tissue culture flasks[1] thoroughly with ice cold buffers: twice with "Washing Buffer I" (5 mL / flask) and once with "Washing Buffer II" (5 mL / flask).

ii- Detach the fibroblasts in chilled "Isolation buffer" (3 mL / flask) using a rubber policeman.

iii- Collect the cells by centrifugation at 600 x g, for 5 minutes, at 4°C. Resuspend all the pellets in a total volume of 3 mL "Isolation buffer".

---

[1] The average number of tissue culture flasks used per fractionation is 20 (75cm2) flasks

iv- Cell disruption is performed on ice. The homogenizer is cooled prior to use. Break the cell pellet by 20-30 strokes, using the tight fitting pestle B of a glass homogenizer. Pipet 1 mL of the homogenate for total enzyme activities and protein determination.

v- Remove nuclei and broken cells by centrifugation at 600 x g, for 10 minutes at 4°C.

vi- Keep the supernate, subject the pellet to 10 additional strokes, and repeat centrifugation.

vii- Layer the post nuclear supernates on top of 10 mL 25% Percoll gradient (p=1.065). The gradient is underlayered with 0.7 mL 2.5 mol/L sucrose. Centrifuge at 48,200 x g in a JA20 rotor, for 60 minutes at 4°C.

viii- Collect 1 mL fractions from the top of the tube for enzyme and protein assays.

3. Protein determination

Quantitation of protein in the fibroblast homogenates was performed according to a modification of Lowry's method; whereas the protein content in the lysosomal fractions was determined according to a method described by Khan et al (1981).

3.1 Protein determination in fibroblast homogenate: Modification of Lowry

Principle

Protein interacts initially with copper in alkali; then the Cu-protein complex, the tyrosine, and the tryptophan of the protein reduce phosphotungstic and phosphomolybdic acids (Folin Ciocalteau reagent) to tungsten blue and molybdenum blue respectively. The standard curve ranges from 30 µg to 150 µg and the absorbance is measured at 750 nm.

Reagents

1) Bovine Serum Albumin

Dissolve 30 mg Bovine Serum Albumin (Sigma A-4503) in 100 mL deionized water. Store at 4°C no longer than 2 months.

2) Solution A

Dissolve 2 g Potassium Sodium Tartarate, and 100 g Na_2CO_3 in 400 mL deionised water. Add 500 mL 1 mol/LNaOH, mix well, and qs to 1000 mL with deionized water. Shelf life is 6 months at room temperature.

3) Solution B

Dissolve 2 g Potassium Sodium Tartarate, and 1 g $CuSO_4.5H_2O$ in 50 mL deionized water. Add 10 mL 1 mol/L NaOH, mix well, and qs to 100 mL with deionized water. Shelf life is 6 months at room temperature.

4) Solution C

Dilute 1 volume of Folin-Ciocalteau reagent (2N Phenol reagent) with 15 volumes of deionized water just prior to use.

Procedure

i- Prepare Bovine Serum Albumin standards as follows:

Table 2.1 **Bovine serum standard (Lowry's method)**

Serum bovine albumin (mg / tube)	Serum bovine albumin (mL / tube)	Deionized water (mL / tube)
0.00	0.00	1.00
0.03	0.10	0.90
0.06	0.20	0.80
0.09	0.30	0.70
0.12	0.40	0.60
0.15	0.50	0.50

ii- Dilute 50 µL of fibroblast homogenate with 950 µL deionised water. Set up a protein control .

iii- To all tubes add 0.45 mL of Solution A. Mix well.

iv- Incubate all the tubes at 50°C in a water bath for 10 minutes. Remove and cool to room temperature.

v- To all the tubes add 50 µL of Solution B. Mix well, and let stand at room temperature for 10 minutes.

vi- Add 1.5 mL of Solution C to all the tubes and mix vigorously.

vii- Repeat incubation at 50°C for 10 minutes, then cool to room temperature.

viii- Read absorbance at 750 nm against reagent blank.

ix- Plot absorbance versus concentration in mg / tube.

Calculations:

mg (from the graph) x 20 = mg protein / mL

3.2 Protein quantitation in lysosomal fractions in the presence of Percoll

Principle

The method is based on measuring the differences in the extent of the absorbance at 750 nm and 420 nm of the color which develops by protein plus Percoll with Folin-Ciocalteau reagent (Khan et al, 1981).

Reagents

1) 2% Na_2CO_3

Dissolve 10 g of Na_2CO_3 in 500 mL deionized water.

2) 2% Sodium-Potassium Tartarate

Dissolve 2 g of Sodium-Potassium Tartarate in 100 mL deionized water.

3) 1% $CuSO_4$

Dissolve 1 g of $CuSO_4$ in 100 mL deionized water.

These stock solutions can be stored at 4°C for six months. The copper reagent is prepared just prior to use, by mixing 98 parts 2% Na_2CO_3 with 1 part 2% Sodium-Potassium Tartarate and 1 part 1% $CuSO_4$.

4) 1.2 mol/L NaOH

Dissolve 4.8 g of NaOH in 100 mL deionized water.

5) The Folin-Ciocalteau reagent (2N)

Dilute one volume of the reagent with one volume of deionized water just prior to use.

6) 0.5 mol/L sucrose

Dissolve 17.12 g sucrose in 100 mL deionized water.

7) 0.25 mol/L sucrose

Dissolve 8.56 g sucrose in 100 mL deionized water.

Procedure

Use 15 x 120 mm disposable culture tubes.

i-Prepare standard curve of bovine serum albumin (20-100 µg) in 0.25 mol/L sucrose (total sample volume = 500 µL).

Table 2.2 **Bovine serum albumin standards (Khan, 1981)**

Bovine serum albumin (µg / tube)	Bovine serum albumin (µL / tube)	Deionized water (µL / tube)	0.50 mol/L sucrose (µL / tube)
20	20	230	250
40	40	210	250
60	60	190	250
80	80	170	250
100	100	150	250

ii- Prepare Percoll standards

A 25% Percoll solution is prepared in 0.25 mol/L sucrose (v/v).

Table 2.3 **Percoll standards**

Percoll concentration (%)	25% Percoll solution (µL)	0.25 mol/L sucrose (µL)
5%	100	400
10%	200	300
15%	300	200
20%	400	100
25%	500	0.00

iii- Dilute the samples (the subcellular fractions) with 0.25 mol/L sucrose 1:1, total sample volume = 500 µL

iv- Prepare reagent blanks :

a) 500 µL deionized water, b) 500 µL 0.25 mol/L sucrose

v- Add to all the tubes 500 µL of 1.2 mol/L NaOH and mix well. White turbidity is observed in all tubes containing Percoll.

vi- Heat all the tubes in a boiling water bath for 20 minutes (cover the tubes with marbles to avoid evaporation). The white precipitate disappears.

vii- Add 5 mL of the Copper reagent to all the tubes. Mix well and incubate at room temperature for 20 minutes.

viii- Add 500 µL of the diluted Folin-Ciocalteau to all the tubes. Mix vigorously, and incubate at room temperature.

ix- Read the absorbances at 750 and 420 nm within 60 minutes of the final color development.

Calculations:

Plot the standard graphs of both the Percoll and the bovine serum albumin; m_1, m_2, m_3, m_4, and C_1, C_2, C_3, C_4 are the slopes and ordinate intercepts, respectively, of the straight lines.

Protein concentration (μg / sample) = $(A_{750} - \alpha A_{420} + \beta) / m_1 - \alpha m_3$

where,

A_{750} = absorbance rate at 750 nm

A_{420} = absorbance rate at 420 nm

$\alpha = m_2 / m_4$

$\beta = \alpha C_3 + \alpha C_4 - C_1 - C_2$

4. Marker Enzyme Assays

Marker enzyme activities: β-hexosaminidase for lysosomes, succinate dehydrogenase for mitochondria, and 5'nucleotidase for plasma membranes are assayed in fibroblast homogenates and subcellular fractions.

4.1 β-hexosaminidase enzyme (Lysosomal marker):

Principle

The assay is performed by adding the sample i.e. a homogenate of fibroblasts or a subcellular fraction, to the buffered substrate, p- nitrophenyl-β-D-N-acetyl glucosaminide. β-hexosaminidase enzyme in the sample hydrolyses the nitrophenyl derivative and p-nitrophenol is released. The latter is measured colorimetrically at 400 nm at an alkaline pH (Pisoni et al, 1985).

The lysosomal membrane integrity is determined by measuring the enzyme latency i.e. the enzyme activity in the presence and absence of a detergent such as Triton X-100. The percentage of broken lysosomes is represented by the free β-hexosaminidase activity (measured in the absence of the detergent), expressed as a percentage of the total enzyme activity (measured in the presence of the detergent) (Rome and Crain, 1981).

Reagents:

1) 0.034 mol/L Citric acid - 0.11 mol/L Phosphate Buffer pH 4.9

Dissolve 4.13 g Citric acid (anhydrous MW= 197.13) and 7.18 g Na_2HPO_4 (MW= 141.96) in 450 mL deionized water. Adjust pH to 4.9 with 1N HCl and q.s. with deionized water to 500 mL. The buffer can be stored at 4°C for 3 months.

2) 4 mmol/L p-Nitrophenyl β-D-N-acetyl glucosaminide pH 4.9

Dissolve 0.137 g p-Nitrophenyl β-D-N-acetyl glucosaminide (MW= 342.3) in 100 mL citric acid - phosphate buffer pH 4.9. Keep frozen in aliquots of 10 mL each at -20°C. Do not freeze and thaw more than twice.

3) 0.8 mol/L Glycine / NaOH buffer pH 10.4

Dissolve 30.03 g Glycine (MW= 75.07) in 450 mL deionized water. Adjust pH to 10.4 with 10N NaOH and q.s. with deionized water to 500 mL.

4) 10 mmol/L p-Nitrophenol (Stock standard solution)

Dissolve 0.139 g p-Nitrophenyl (MW= 139.11) in 100 mL glycine / NaOH buffer pH 10.4.

5) p-Nitrophenol (Working standard solution)

Dilute 0.5 mL stock solution with 50 mL glycine / NaOH buffer

6) 0.1% Triton X-100

Prepare a 10% solution of Triton X-100 and and use a dilution. Dissolve 10 g Triton X-100 in 100 mL deionized water

Procedure:

i-Prepare standards

***Table 2.4* p-Nitrophenol standards**

Working standard (µmol / tube)	Working standard (mL / tube)	Glycine / NaOH buffer (mL / tube)
0.00	0.00	2.00
0.01	0.10	1.90
0.02	0.20	1.80
0.04	0.40	1.60
0.05	0.50	1.50
0.10	1.00	1.00
0.20	2.00	0.00

ii- Prepare two sets of tubes . Label one set with "T". Add 50 µL sample to all the tubes.

iii- Add 20 µL 10% Triton X-100 to the labeled set of tubes.

iv- Add 450 µL substrate to all the tubes. Incubate at 37°C for 15 minutes.

v- Stop the reaction by adding glycine / NaOH, 1.48 mL to the "T" labeled tubes and 1.5 mL to the other set of tube; total volume = 2 mL

vii- Read absorbance at 400 nm against substrate blank.

Calculations:

One unit of β-hexosaminidase activity is defined as 1 nmol of p-nitrophenol formed per minute at 37°C (Pisoni et al, 1985).

Plot a standard curve.

The reading from the graph = µL p-nitrophenol / Tube / 15 minutes.

The reading from the graph x 20 /15 = µL p-nitrophenol / min / mL at 37°C

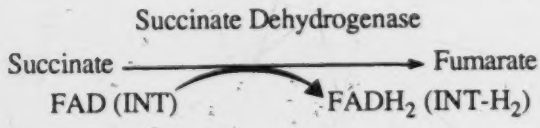
Therefore,

IU / mL = nmol p-nitrophenol / min / mL at 37°C

4.2 Succinate dehydrogenase (Mitochondrial marker):

Principle

The assay is carried out by adding the sample i.e. a homogenate of fibroblasts or a subcellular fraction, to the substrate, sodium succinate, in the presence of p-iodophenyl-3-p nitrophenyl-5-phenyl tetrazolium chloride (INT). Formazan is extracted by ethyl acetate and its absorbance is measured at 490 nm (Pennington, 1961).



Reagents:

1) 50 mmol/L Potassium Phosphate Buffer, pH 7.4

- Solution A:

Dissolve 0.689 g KH_2PO_4 (monobasic) in 100 mL deionized water

- Solution B:

Dissolve 0.870 g K_2HPO_4 (dibasic) in 100 mL deionized water

To make up Potassium Phosphate Buffer pH 7.4 add 19 mL of Solution A to 81 mL of Solution B and mix well.

The substrate consists of :

50 mmol/L Potassium Phosphate Buffer pH 7.4	100 mL
25 mmol/L Sucrose	0.856 g
50 mmol/L Sodium Succinate	1.350 g
0.1% INT	0.100 g

The substrate is stable for 2 weeks at -20°C. It is preferred to add the INT just prior to use.

Procedure:

i- Add 100 µL sample to 900 µL substrate.

ii- Shake the reaction mixture gently at 37°C for 15 minutes.

iii- Stop the reaction with 1 mL 10% Trichloroacetic acid.

iv- Extract formazan by adding 4 mL ethyl acetate. Vortex for 30 seconds.

v- Centrifuge at 800 x g for 10 minutes at room temperature.

vi- Remove an aliquot of the supernatant and measure absorbance at 490 nm versus substrate blank.

Calculations:

The molar extinction coeffecient of formazan in ethyl acetate at 490 nm is 20.1 x $(10)^3$

1 mol/L $\varepsilon = 20.1 \times (10)^3$

1 mmol/L $\varepsilon = 20.1$

$OD = \varepsilon C l$

where,

ε = extinction coeffecient

C = concentration

l = length = 1 cm.

Therefore,

$C = OD / \varepsilon l$

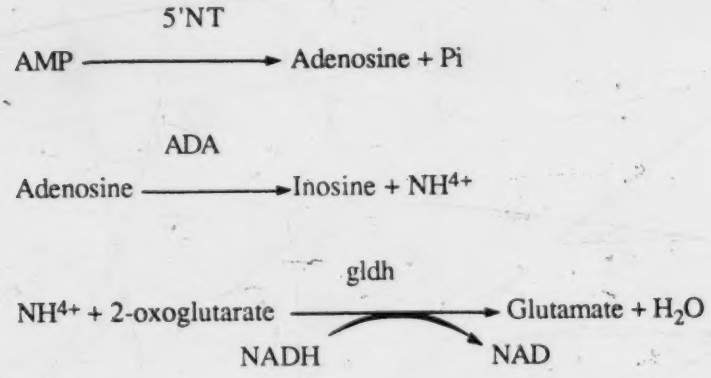
C = OD/20.1 x 4/1 x 1/15 µmol/min/4 mL

C = OD/20.1 x 4/15 x 1/mg µmol/min/mg protein

4.3 5'nucleotidase enzyme (plasma membrane marker):

Principle

5'nucleotidase catalyses the hydrolysis of adenosine monophosphate (AMP) to give adenosine and inorganic phosphorus. Adenosine is deaminated by adenosine deaminase (ADA) to produce ammonia, which in turn reacts with 2-oxoglutarate and NADH, which is oxidised to NAD (Arkesteijn, 1976) as follows:



The rate of formation of NAD is followed automatically on a Multistat III microcentrifugal analyzer by measuring the decrease in absorbance at 340 nm which is directly proportional to the 5'nucleotidase activity. β-glycerophosphate is added to inhibit alkaline phosphatase activity.

5. Amino acid Analysis

5.1 Principle

Amino acid content of the fibroblasts homogenate and the lysosomal fractions is measured in protein-free-filtrates by High Pressure Liquid Chromatography on a cation exchange column. Continuous post-column derivatization of the amino acids is achieved with o-phthalaldehyde (OPA) in conjunction with a mercaptan under basic conditions. At ambient temperature, the reaction is generally complete within 30 seconds. The fluorescent derivatives, 1-alkylthio-2-alkylsubstituted isoindoles exhibit optimum excitation at 340 nm and emission at 455 nm (Yakymyshyn and Audette, 1990; Yakymyshyn, 1991).

5.2 Reagents

1) 0.24 mol/L Lithium Citrate Eluent (LI 275) Buffer, pH 2.75

2) 0.64 mol/L Lithium Citrate Eluent (LI 750) Buffer, pH 7.50

3) 0.30 mol/L Lithium Hydroxide Column Regenerant (LI Regen)

4) 0.27 mol/L Lithium Citrate Diluent , pH 2.2

5) 10 mmol/L β- Thienyl-alanine stock internal standard

6) Working Internal standard / buffer mixture:

Dilute 0.60 mL stock β-Thienyl-alanine standard with 100 mL Lithium Citrate diluent, pH 2.2.

7) 10 mmol/L L-Glutamine:

Dissolve 14.6 mL L-Glutamine in 10 mL Lithium Citrate diluent, pH 2.2

8) 2.5 mmol/L L-Tryptophan and 1.25 mmol/L L-Asparagine:

Dissolve 51.0 mg L-Tryptophan and 18.8 mg L-Asparagine in 100 mL Lithium Citrate diluent, pH 2.2

9) Physiological Calibration Standard, stock solutions , 2.5 mmol/L

Beckman STD #338088

Beckman AN^+ #338156

Beckman B^+ #338157

10) 250 µmol/L Working Calibration Standard

Dissolve 1 mL of Beckman STD, 1 mL Beckman Standard AN^+, 1 mL Beckman Standard B^+, 1 mL Tryptophan / Asparagine Standard, and 0.25 mL L-Glutamine in 10 mL Lithium Citrate diluent, pH 2.2

11) o-Phthalaldehyde Fluorimetric Reagent

12) Sulfosalicylic acid 30 mg/mL sample

13).Column Cation Exchange : 3 mm x 150 mm, 7 µm

14).Millipore Milex Filters, 0,45 µm pore size

15) Amicon membrane cones cF50 (molecular weight cut off is 50,000)

5.3 Instrumentation

Instrumentation consists of a Perkin-Elmer HPLC adapted with the Pickering Labs Amino Acid Application. The Perkin-Elmer modular instrument consists of a 4-Solvent Titanium LC410 Biopump providing the continuous gradient of lithium citrate to the column at a rate of 0.30 mL / min, an ISS-100 automated sample injector fitted with a 20 mL sample loop, a Series10 HPLC isocratic pump for continuous delivery of o-phthalaldehyde at a rate of 0.20 mL / min for postcolumn derivatization at ambient temperature, an LS-5 luminescence spectrometer to measure fluorescence of the amino acid derivatives,1-alkylthio-2-alkylsubstituted isoindoles, at their excitation and emission optima of 340 nm and 455 nm, respectively, with slits of 10 and a fixed scale setting of 2.0. An LCI-Laboratory computing integrator provides a hard copy of the resultant chromatograms with calculations of each constituent amino acid. A calibration standard of known concentration and a control sample are run with each set of samples to ensure accuracy and precision of results. The analytical detection limit is as low as 1 pmol for individual amino acids.

5.4 Preparation of the samples for amino acid analysis

i- Pool the lysosomal fractions (highest β-hexosaminidase activity). Wash the fractions with ultrafiltration using Centriflo membrane cones cF50 (molecular weight cut off 50,000). The membrane cones should be soaked in deionized water for at least one hour prior to use. Filter the samples through the cones by centrifugation at 3000 rpm in a Sorvall

centrifuge at 4°C for 30 minutes. Wash the retentate with 1.5 mL phosphate buffered saline and repeat the filtration. Resuspend the retentate in 500 µL phosphate buffered saline and disrupt the lysosomes by 0.1% Triton X-100, and immediately acidify with sulfosalicylic acid (30 mg / mL sample) and repeat the filtration for the last time. Store the filtrates at -70°C.

ii- At time of analysis thaw and filter the samples through a 0.45 µm pore size millipore HV filter (CAT#SJHV004NS) to remove any residual protein.

iii- Dilute 19.5 µl filtrate with 5 µl 0.27 mol/L lithium citrate buffer pH 2.2, containing β-thienylalanine as internal standard.

5.5 Procedure

i- The equivalent of 19.5 µL of sample in a total volume of 20 µL is injected onto a High Speed Lithium Cation Exchange Column, 3 mm x 150 mm, 5 µm (Pickering Labs).

ii-The amino acids are separated using a continuous gradient of 0.24 to 0.64 mol/L lithium citrate pH 2.75 to 7.50, respectively. Regeneration of the column is performed with 0.30 mol/L lithium hydroxide. Run time for a complete amino acid profile is 136 minutes. Composition of the buffer gradient used for the separation of amino acids is given in *(Table 2.5)*.

Table 2.5 **Composition of the buffer gradient used for the separation of amino acids**

Step	Time	Flow	% A LI 2.75	% B LI 7.50	% C	% D LI Regen	Curve
0[1]	20.0	0.30	100	-	-	-	-
1	9.0	0.30	100	-	-	-	-
2	37.0	0.30	65.0	35.0	-	-	1.0
3	44.0	0.30	-	100	-	-	1.0
4	6.0	0.30	-	100	-	-	-
5	30.0	0.30	-	94.0	-	6.0	2.5
6	5.0	0.30	-	80.0	-	20.0	4.0

Calculations

Concentration of a specific amino acid =

Amino acid concentration in the calibration standard X $\frac{\text{Peak height of the amino acid in the sample}}{\text{Peak height of the amino acid in the calibration standard}}$

[1] Step "0" is the equilibration step following regeneration prior to injection

In contrast to peak heights, peak areas did not give a linear response with different concentrations of various amino acids.

It is important to note that cystine content has been conventionally reported as "1/2 cystine" because routine subnanomolar quantitation of cystine was performed by ion exchange chromatography with postcolumn identification by ninhydrin staining. This procedure measures total cystine plus cyst(e)ine and cannot differentiate the two and therefore the amount of disulfide has been expressed as half cystine.

6. Electron microscopy

The purity of the isolated lysosomal fractions was judged by assaying marker enzyme activities and by electron microscopy.

Procedure:

6.1 Preparation of samples

a- whole fibroblast

A cell pellet of normal fibroblasts (GM00010) is fixed overnight at 4°C in 4% gluteraldehyde solution in cacodylate buffer, in eppendorf tubes.

b- lysosomal fractions

Pool the lysosomal fractions into two groups the upper fractions, the light lysosomes (p = 1.049-1.054), and the lower fractions, the heavy lysosomes (p = 1.096-1.107). Fix the samples with 1% glutaraldehyde-1% formaldehyde in 0.1mol/L cacodylate for one hour at room temperature. Centrifuge the samples at 35,000 x g for 30 minutes at 4°C. Remove the supernate, rinse and store the pellets overnight at 4°C in 0.1 mol/L cacodylate buffer in eppendorf tubes (Oliver et al, 1989).

6.2 Specimen processing:

i- Fix the samples (a and b) in 1% OsO_4 for two hours at 4°C, with the pellet remaining at the bottom of the ependorff tubes. Rinse the pellets once with cold cacodylate buffer, and then with deionized water for 5 minutes each.

ii- Cut the the eppendorf open with a razor, remove the pellet, cut it into 3 mm^3 portions, and place in deionized water.

iii- Dehydrate the portions in a graded series of ethanol solutions, then rinse three times in propylene oxide.

iv- Place the portions in a 50 : 50 mixture of propylene oxide: epon for 30 minutes, and then in pure epoxy resin (Epon 812) for 2 hours.

v- Place the portions in embedding capsules with fresh epoxy resin and cure them at 60°C for 48 hours.

In case of the heavy lysosomal fraction, where the collected material can be extremely small, dehydrate the pellet and infiltrate it with epon while still in the ependorff tube. Cut the tube open and scrape the bottom to remove the material. Place the scrapped material in an embedding capsule filled with epon. Centrifuge at 2000 x g for 30 minutes.

vi- Remove the various blocks from the capsules and thick section them. Mount the thick (1 μm) sections on glass slides, stain with Toluidine Blue, and examine with the light microscope.(The block consisting of the extremely small material was only thin sectioned to avoid sectioning away the material).

vii- Select a block from each specimen, further trim, and thin section (100 nm) for electron microscopy examination. Mount the thin sections on grids and contrast them with Uranyl Acetate and Lead Citrate.

viii- Examine the grids with the electron microscope (Hitachi 600).

Chapter Three: Results

1. Tissue culture

It was observed that mutant cell lines, cystinotic and particularly, I-cell fibroblasts, grew much slower compared to normal fibroblasts, and that their subculture was only possible at a ratio of one to two flasks. It was also found that I-cell fibroblasts were very sensitive to freezing, and when salvaged from liquid nitrogen, they required a significantly longer period of time than controls to reach confluency.

2. Subcellular fractionation of human fibroblasts and marker enzyme assays

The subcellular fractionation of human skin fibroblasts was based on the method described by Gieselmann et al (1983); however, some modifications were made. Several lysis techniques were attempted: sonication, passing the cell suspension through a pipette tip (1.45 mm in diameter) 15 times, and homogenization in a glass homogenizer. The degree of cell lysis was assessed by examination under light microscope. It was found that sonication resulted in inconsistent lysis, and passing cell suspensions through the pipette tip was not disruptive enough to break the cell membranes. Homogenization was found to be the preferred lysis technique. However, it was observed that the number of strokes needed to be varied between different cell lines to achieve the same degree of disruption. Mutant cell lines, especially, I-cell fibroblasts must be subjected to gentler homogenization i.e.10 strokes instead of 20. This also seemed to apply to ageing normal fibroblasts. This conclusion was arrived at from two sets of data: 1) loss of β-hexosaminidase latency with excessive homogenization of mutant cell lines, indicating that the stroking is too strong and disrupts the lysosomal membrane integrity e.g. β-hexosaminidase activity measured in the absence and presence of Triton X-100 in the I-cell fibroblast (GM02013B) homogenate was 39.5 IU/mL and 61.1 IU/mL, respectively, which indicates that the lysosomal membrane integrity was conserved only to the extent of 35%, 2) high β-hexosaminidase activity in the pelleted broken membranes and nuclei indicates the presence of unbroken cells e.g. β-hexosaminidase activity in the control fibroblast homogenate and in pelleted broken membranes and nuclei was 79.6 and 151.6 IU/mL, respectively, in the absence of Triton X-100 and 312.0 and 448.2 IU / ml, respectively in its presence.

Centrifugation of the postnuclear supernates layered on a 25% Percoll gradient yielded two peaks of lysosomal marker enzyme activity (β-hexosaminidase). The activity recovered in the lower density fractions (p= 1.049 - 1.054) was found to be between 20-50% of that recovered in the higher density region (p= 1.096 - 1.107). β-hexosaminidase activity in I-cell disease fibroblasts was significantly lower than control values (10 - 17% of

normal) i.e. β-hexosaminidase activity in normal and I-cell fibroblast homogenates in the presence of Triton X-100 was 434.7 and 72 IU / ml, or 6.86 and 1.18 μmol/hr/mg protein, respectively.

Lysosomal membrane integrity was conserved to the extent of 75 - 85% in most of the granular fractions as judged by the latency of β-hexosaminidase activity. The latter was determined by the difference in β-hexosaminidase activity in subcellular fractions of normal human fibroblasts (GM00010) in the presence and absence of Triton X-100 (*Fig 3.1*).

The heavy lysosomal population sedimenting from human skin fibroblasts, was found to be relatively free of enzyme activities specific to other intracellular organelles. The marker enzymes succinate dehydrogenase for mitochondria and 5'nucleotidase for plasma membranes were present to a significant degree only in the buoyant fractions near the top of the Percoll gradient (*Fig 3.2*).

3. Electron microscopy

Results of the electron microscopic examination of the whole fibroblast pellet *(micrograph a)* reveals a tightly packed arrangement of cells which show little evidence of freeze artifact. Cells show pleomorphic nuclei (N) and cytoplasm revealing features typical of fibroblast cells, namely dilated rough Endoplasmic Reticulum (ER) cysterna, lipid droplets, mitochondria (M) and few lysosomes (L) in a filamentous matrix. Cells showed secondary lysosomes, and a few forming lysosomes with contents of low electron density were noted surrounded by tiny vesicles probably arising from Golgi Apparatus.

Sections of the upper lysosomal fractions *(micrograph b)* showed a mixture of mitochondria, ER, ribosomes, and lysosomes, both primary and secondary. Whereas in the lower lysosomal fractions *(micrographs c, d)* very few structures were noted ("solid arrow" points to intact lysosomes whereas "empty arrow" points to disrupted lysosomes). Most of these structures had characteristics of primary lysosomes: i.e. very electron dense contents surrounded by a single limiting membrane. The remainder of the structures showed lamellar appearance and membranous interiors typical of secondary lysosomes.

4. Protein Determination

The harvest of twenty confluent 75 cm^2 flasks (5.0 x 10^6 cells / flask) generally, yielded about 4 mg protein / mL homogenate sample. Problems were encountered in determining the protein concentration of the gradient fractions. When the modified Lowry method was used, there was an overestimation of the protein content due to the presence of Percoll which forms a colour with the Folin reagent. The Bio-rad assay was also tried but did not give satisfactory results due to the intense turbidity which immediately formed

when Percoll was present in the samples. Precipitating the protein with deoxycholate then redissolving it in NaOH was also found unsuccessful due to the formation of turbidity in the samples containing Percoll. Finally, the method described by Khan et al, (1981), for the determination of protein in the presence of Percoll was used. In this method, the Percoll colour interference is corrected for. Standard curves for protein and Percoll at absorption 750 nm and 420 nm were determined and were found linear i.e. r = 0.989 - 0.998 (*Fig 3.3-3.4*). The differences in the extent of absorbance at 750 and 420 nm of the color developed by protein and Percoll with the Folin reagent are calculated according to certain constants (see methodology). The protein content of the lower and higher density lysosomal fractions ranged from 0.95 to 2.0 mg / ml, and 0.10 to 0.70 mg / ml, respectively.

5. Amino acid analysis

5.1 Preparation of samples for amino acid analysis

Three main problems are encountered in the preparation of the samples for amino acid analysis: a) how to maximally concentrate the lysosomal fractions, b) how to eleminate Percoll from the fractions, and c) how to reduce contamination from amino acids outside the lysosomes.

a) In an attempt to concentrate the gradient fractions for a satisfactory recovery of amino acids, it was necessary to reduce the gradient volume. One third of the gradient volume described by Gieselmann et al (1983) was used while keeping the appropriate column height to ensure the density gradient. This modification has significantly improved the recovery of amino acids in general, and to greater degree the recovery of amino acid cystine. Methods such as lyophilysation, and concentration in a speedvac concentrator were attempted but were found unsatisfactory due to the losses of samples.

b) Separating Percoll from the lysosomal fractions was an important step in the preparation of the samples for amino acid analysis in order not to destroy the column of the HPLC system. Several techniques were tried and the fragility of lysosomes was a constant problem. Lysosomes were separated from the coated silica particles by high speed centrifugation in a swing rotor (at 100,000 x g for 2 hours) or angle head rotor (at 100,000 x g for 90 minutes). The biological particles formed a delicate membrane above a hard Percoll pellet. It was rather difficult to pipet this fragile membrane of biological material without disrupting the lysosomes and losing some of their content. This was assessed by measuring β-hexosaminidase activity in the supernate and in the pelleted biological membrane which were 92.6 and 59.1 IU/mL, respectively. A considerable amount of activity was found in the supernate denoting disruption of lysosomes and loss of latency.

Another trial was done where Percoll was separated by one step of ultrafiltration: lysosomes were disrupted by 0.1% Triton-X-100, acidified with solid sulfosalicylic acid (30 mg / mL sample), and then subjected to ultrafiltration through membrane cones.

c) Finally, to ensure that the filtrate represents the intralysosomal contents, a method which combines several washings of the the granular fractions with phosphate buffered saline (3 volumes buffer to 1 volume sample) together with ultrafiltration through membrane cones, was used. The first filtrate, designated as wash 1, represents two components: the amino acids in solution surrounding the lysosomes and those which might have been released from disruption of lysosomal membranes. Lysosomes are disrupted with Triton X-100, acidified with sulfosalicylic acid, and subjected to a last step of ultrafiltration. The last filtrate is reported as wash 3 and is expected to represent only the intralysosomal contents. The major drawback of these methods was an increased loss of contents from fragile lysosomes during the repeated washing steps. The effect of the washing procedure on amino acid content of I-cell disease lysosomal fractions is shown in *(Table 3.7)*.

In the preliminary trials of amino acid analysis, it was rather difficult to detect cystine in cystinotic cell lines. An important observation was made with regard the recovery of cystine: immediate acidification of samples with sulfosalicylic acid, before storing the samples, gave a better recovery of cystine.

5.2 Problems encountered in amino acid analysis

Typical amino acid chromatograms of normal, cystinotic and I-cell fibroblast homogenates and their lysosomal fractions are given in *(Fig 3.7 to 3.10)*. The system has a high sensitivity with an analytical detection limit as low as 1 picomole for individual amino acids. A considerable number of amino acid peaks are resolved except those of secondary amino acids (proline and hydroxyproline), and the run time for a complete amino acid profile is 136 minutes. One of the major drawbacks observed in this study when running the fibroblast homogenate and gradient fractions, was the occurrence of a shift in the retention times at the beginning of the run compared with the calibration standard *(Fig 3.5a)*. A standard mixture of amino acids was added to the reagent blanks and a similar shifting of retention times was noted *(Fig 3.5b)*. This suggests that the shift in the retention times is due to the conditions of the matrix of the samples. When the pH of the samples injected on the column was tested, it was found within expected ranges i.e.pH < 2. Along with the shift in retention times, incomplete resolution of the acidic amino acid peaks at the beginning of the run was found to occur; these amino acids co-eluted in one big peak. The results of such peaks are reported as "not available" (N/A).

A much larger peak in the position of γ-amino-butyric acid (GABA) was observed in lysosomal amino acid profiles compared with whole fibroblast homogenates *(Fig 3.7 and 3.8)*. GABA content in normal (GM00010) fibroblast homogenate and lysosomal fractions was found to be 0.11 and 46.2 nmol / mg protein, respectively. To clarify whether this was real or artifact due to contamination, different reagent blanks were run, as follows: 1) double distilled water, 2) suspension buffer (0.25 mol/L sucrose and 3 mmol/L imidazole HCl), 3) Percoll gradient made up in double distilled water, 3) phosphate buffered saline, 4) 0.1% Triton X-100, 5) sulfosalicylic acid (30 mg/mL double distilled water), and finally, 6) to test contamination that might arise from membrane cones, a blank of double distilled water was filtered through the membrane cones. A large GABA peak was observed to elute only in the Percoll blank see *(Fig 3.6)*. The reagent blanks showed background amino acids such as glutamic acid, glycine, traces of ornithine, hydroxylysine, histidine, arginine, and other contaminants such as urea and ammonia.

Two other amino acids seemed relatively enriched in the lysosomal fractions compared to whole fibroblast homogenate: glycine and hydroxylysine *(Fig 3.7 and 3.8)*. The average glycine content of normal (GM00010) fibroblast homogenate and lysosomal fractions were found to be 4.26 and 90.20 nmol / mg protein, respectively. There was 1.33 and 32.66 nmol / mg protein hydroxylysine in normal (GM00010) fibroblast homogenate and lysosomal fractions, respectively.

5.3 Amino acid content of Normal, Cystinotic , and I-cell disease fibroblast homogenates

The amino acid content of normal fibroblast homogenates is given in *(Table 3.2)*. The findings of this study show about a ten fold increase in the cystine level in the cystinotic fibroblasts (GM00090A) (7.60 nmol 1/2 cystine / mg protein) *(Table 3.3)*, compared to normal cell lines (GM00010) (0.58 nmol 1/2 cystine / mg protein). However, the cystine content of two other cystinotic cell lines (line# 717 and 1324) showed approximately a five fold increase i. e. 2.30 and 2.80 nmol 1/2 cystine / mg protein. The cystine peak in control samples is noted to be broad *(Fig 3.7a)* and unlike that of the standard amino acid mixture *(Fig 3.5a)*. This could raise some doubt as to the presence of cystine at all.

The amino acid content of I-cell disease fibroblast homogenate is given in *(Table 3.4)*. The cystine content of the I-cell disease whole fibroblast homogenate (GM02013B) was found to be lower than reported values: 1.08 nmol 1/2 cystine / mg protein, and that of line #454 was slightly higher than control values : 0.80 nmol 1/2 cystine / mg protein. The results also demonstrated that in cystinotic and I-cell disease fibroblast homogenates, the

increase in cystine level was unique; other amino acid concentrations were not significantly different from controls.

5.4 Amino acid content of high density lysosomal fractions of normal, cystinotic, and I-cell disease fibroblasts

A comparison of amino acid contents of normal (GM00010) and cystinotic (GM00090A) high density lysosomal fractions is given in *(Table 3.5)*. A significantly high cystine content was found in cystinotic (GM00090A, Line# 1324, and #717) lysosomes (9.20 - 15 nmol 1/2 cystine / mg protein) compared to the control samples, where there was no detectable cystine. Findings of this study showed approximately two fold enrichment of cystine in the cystinotic high density lysosomal fractions when compared with the crude homogenate.

Cystine content in cystinotic fibroblast (line#1324) and heavy lysosomal fractions was compared to that of three other amino acids (valine, leucine, lysine) *(Table 3.6)*. These amino acids showed stable and reproducible peaks.

The amino acid content of lysosomal fractions of I-cell disease fibroblasts are given in *(Table 3.7)*. Only traces of cystine were detected.

Figure 3.1

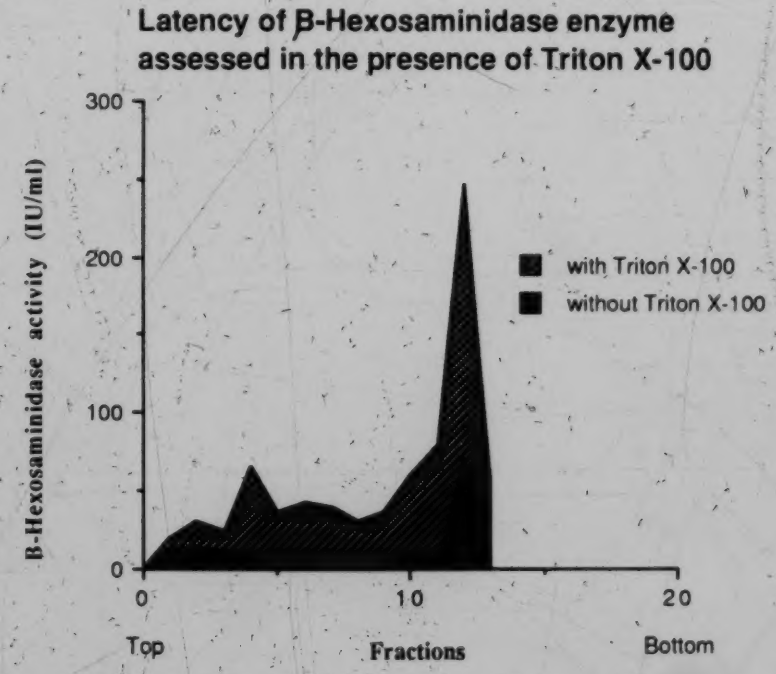
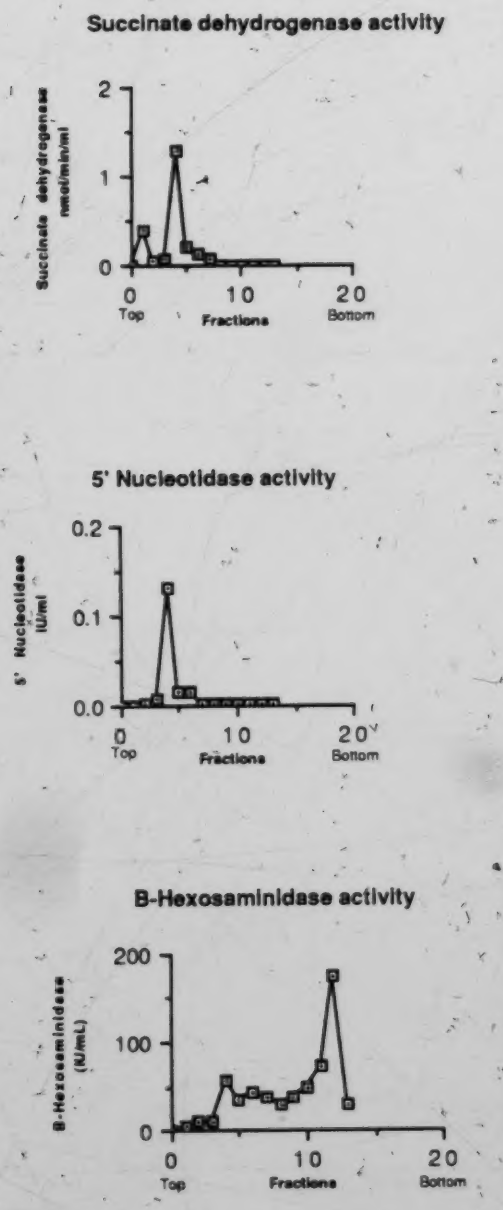
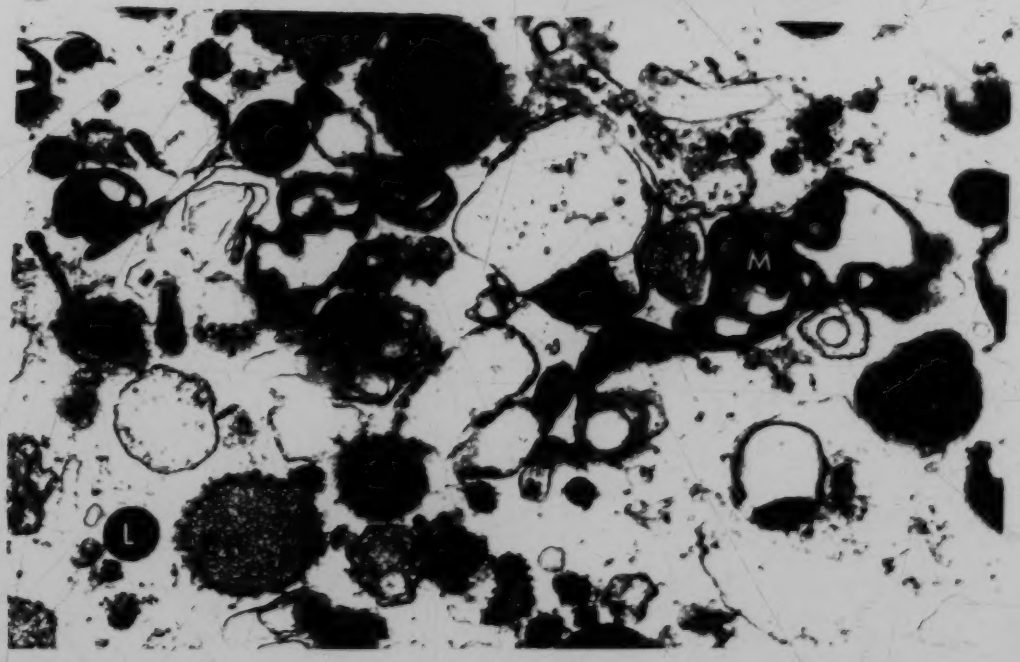


Figure 3.2 Marker enzyme assays



a

b

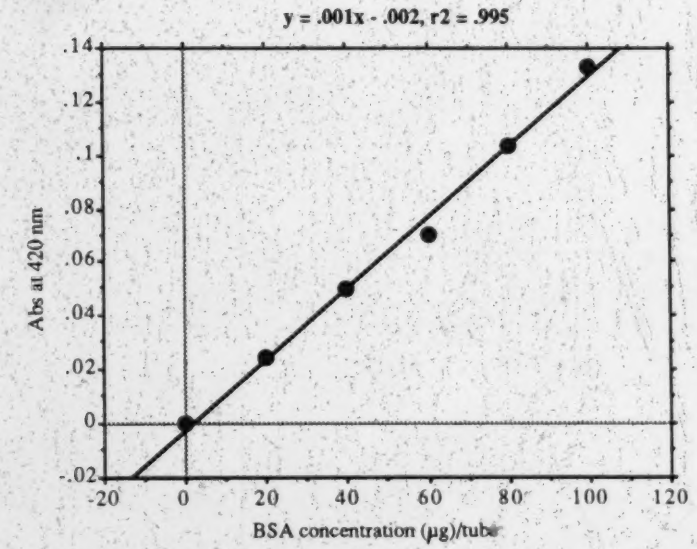


c

d

Figure 3.3 **Bovine serum albumin (BSA) standard curves of absorption at 420 nm (a) and 750 nm (b).**

(a)



(b)

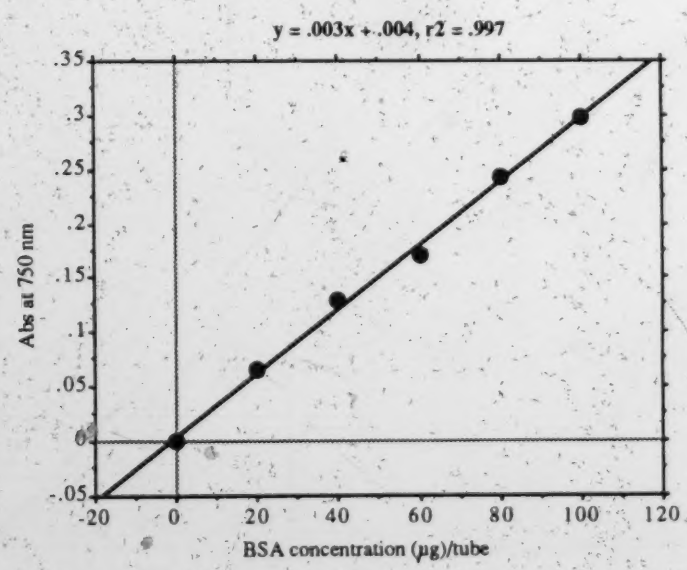
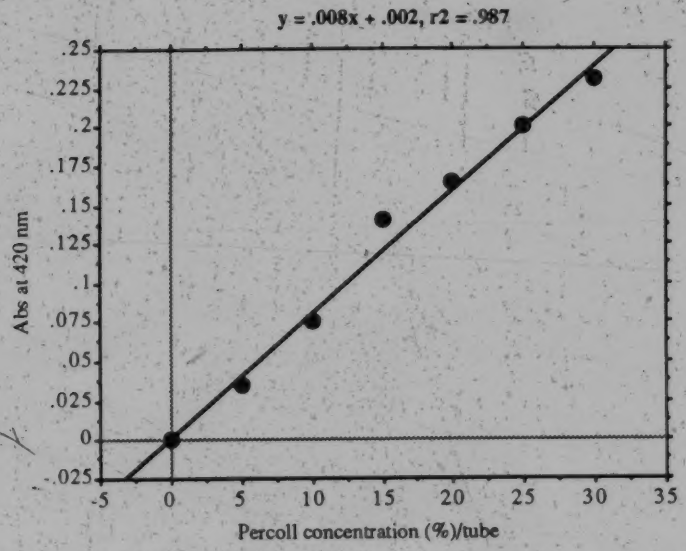


Figure 3.4 **Percoll standard curves of absorption at 420 nm (a) and 750 nm (b)**

(a)



(b)

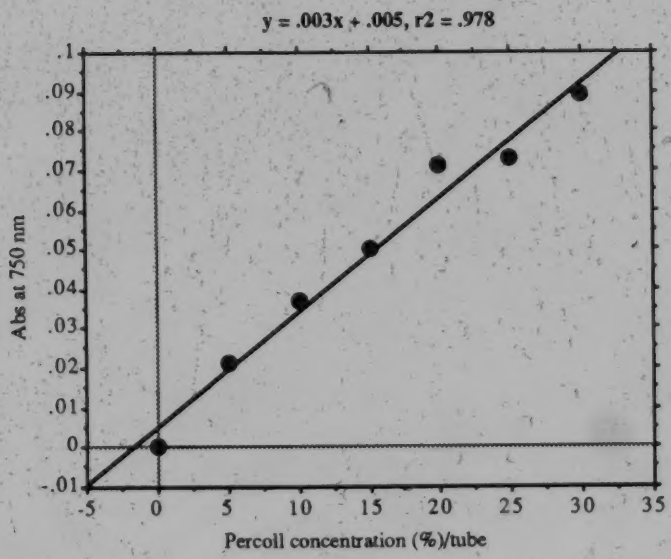


Table 3.1 **Identification of the amino acid peak number (Legend for figures 3.5 to 3.10)**

Peak #	Amino acid	Peak #	Amino acid
1	Taurine	15	Isoleucine
2	Aspartic	16	Leucine
3	Threonine	17	Internal standard
4	Serine	18	Tyrosine
5	Asparagine	19	Phenylalanine
6	Glutamic	20	β-Alanine
7	Glutamine	21	GABA
8	Glycine	22	Tryptophan
9	Alanine	23	Ammonia
10	Citrulline	24	OH-Lysine
11	AABA	25	Ornithine
12	Valine	26	Lysine
13	1/2 Cystine	27	Histidine
14	Methionine	28	Arginine

Figure 3.5 Calibration standard (a). Calibration standard with reagent blank (b)

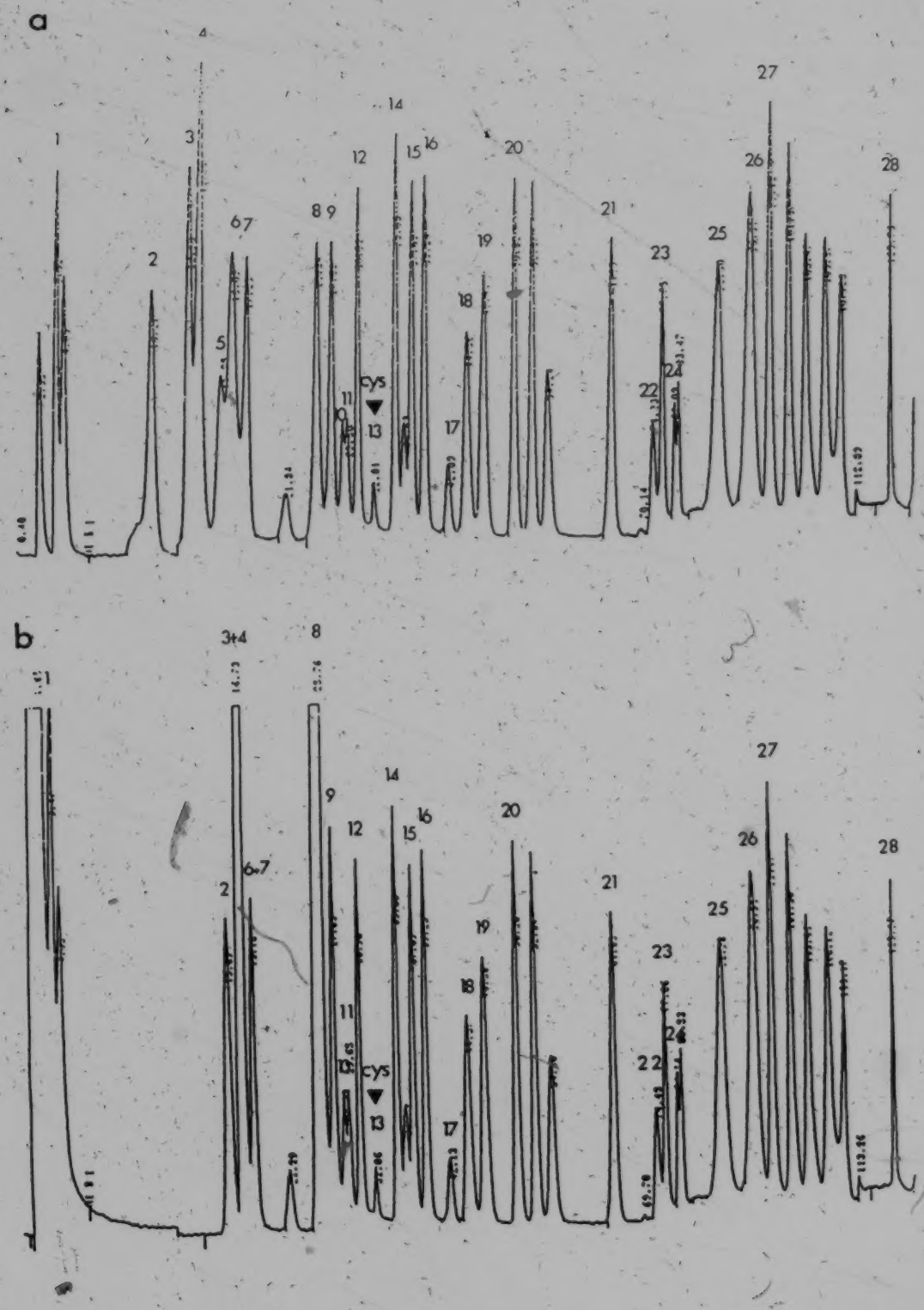


Figure 3.6 **Percoll blank**

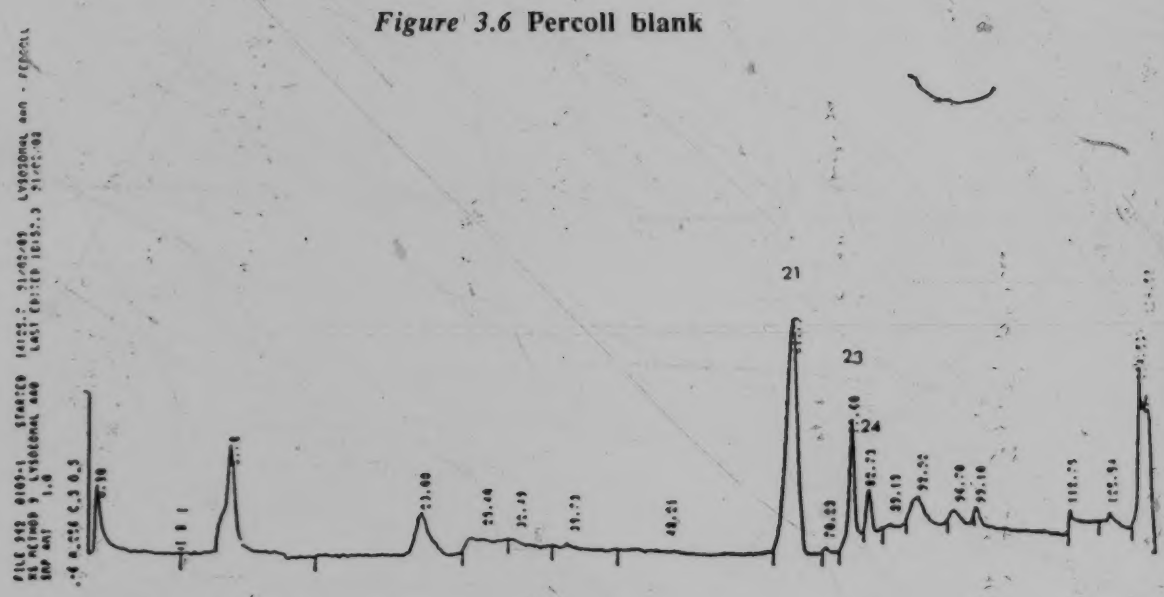


Figure 3.7 Comparison of amino acid profiles in normal (a) and cystinotic (b) fibroblast homogenates

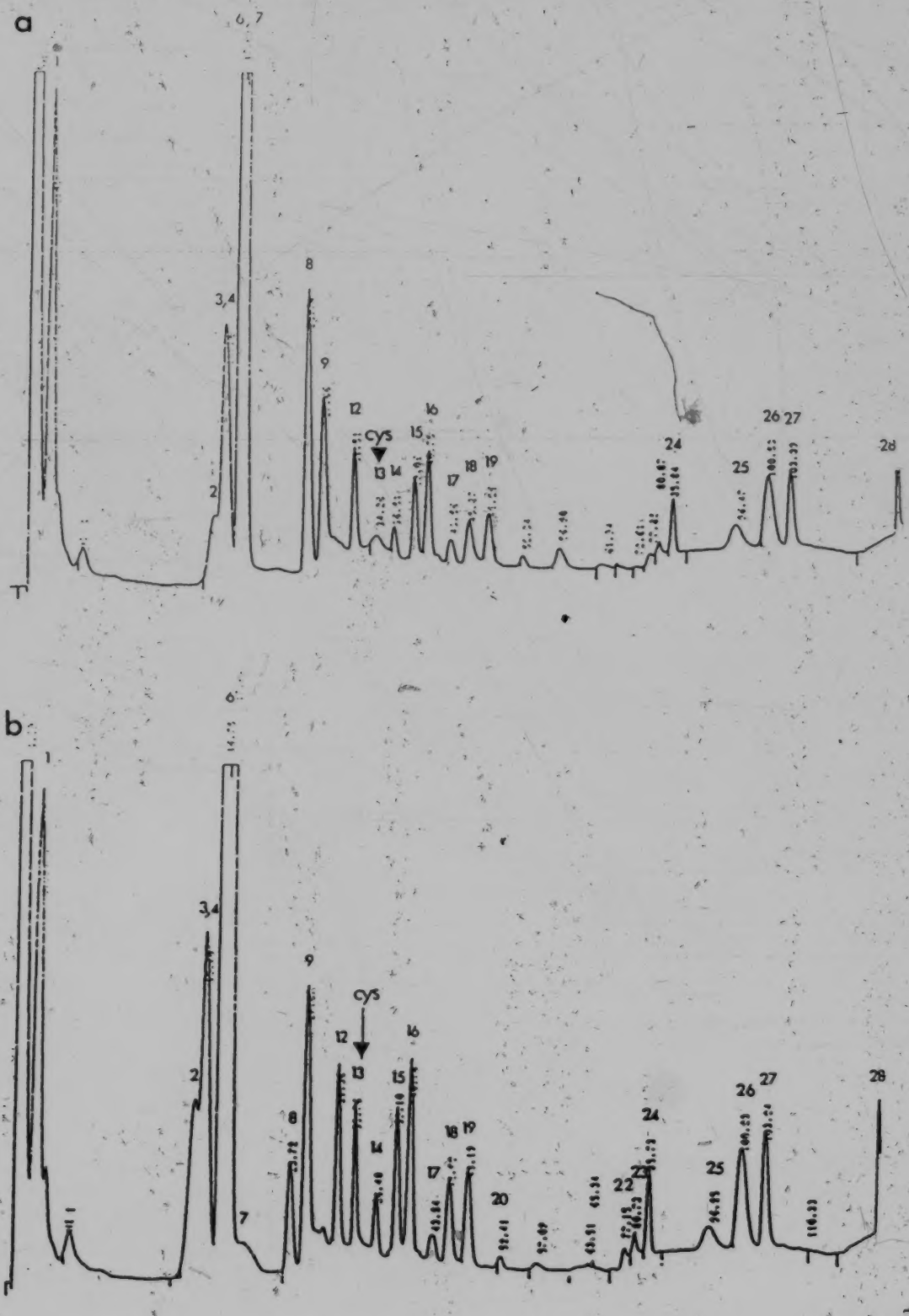


Figure 3.8 **Comparison of intralysosomal amino acid profiles in normal (a) and cystinotic (b) fibroblasts**

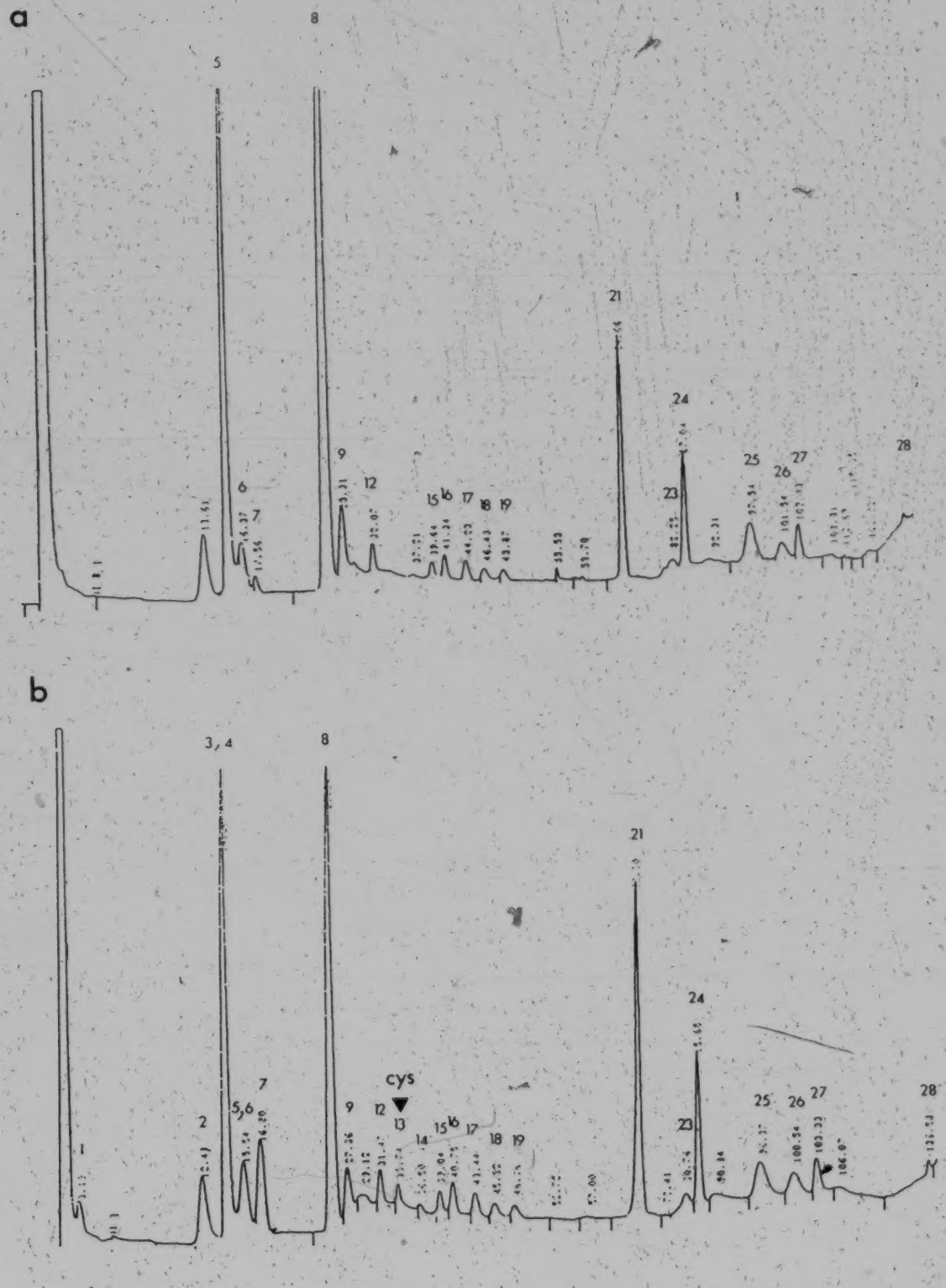


Figure 3.9 **Amino acid profile of I-cell disease fibroblast homogenate**

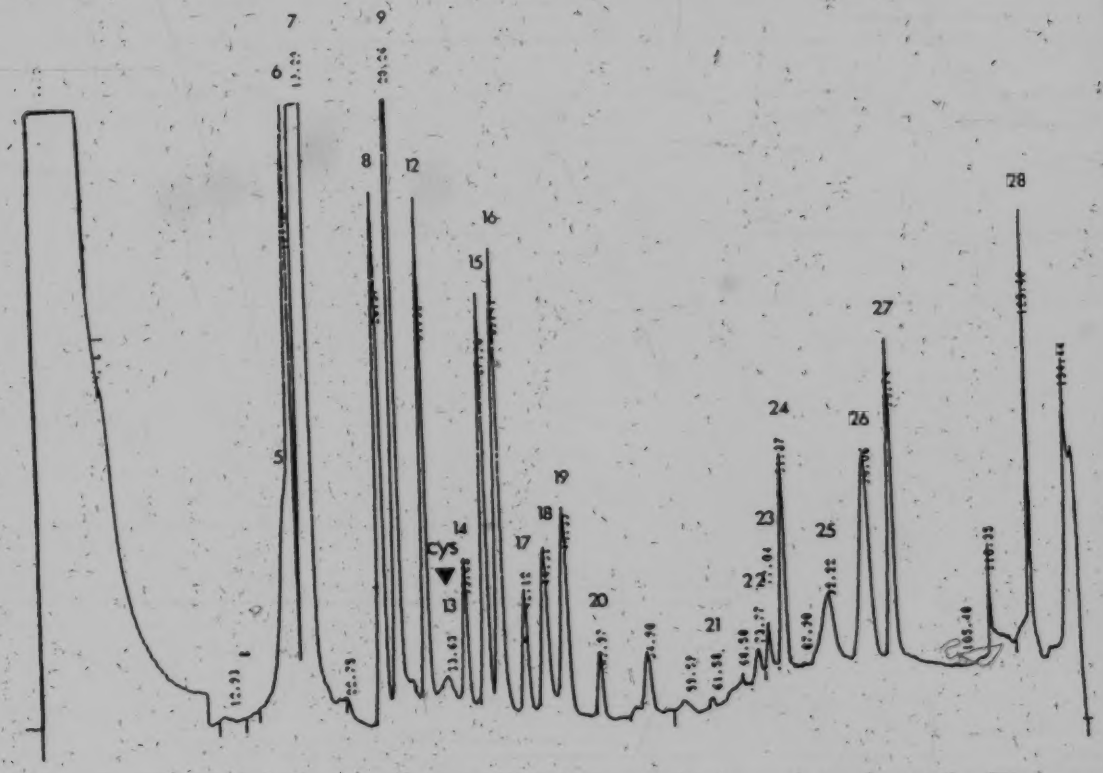


Figure 3.10 **Comparison of intralysosomal amino acid profiles of non-washed (a) and washed (b) fractions of I-cell disease fibroblast**

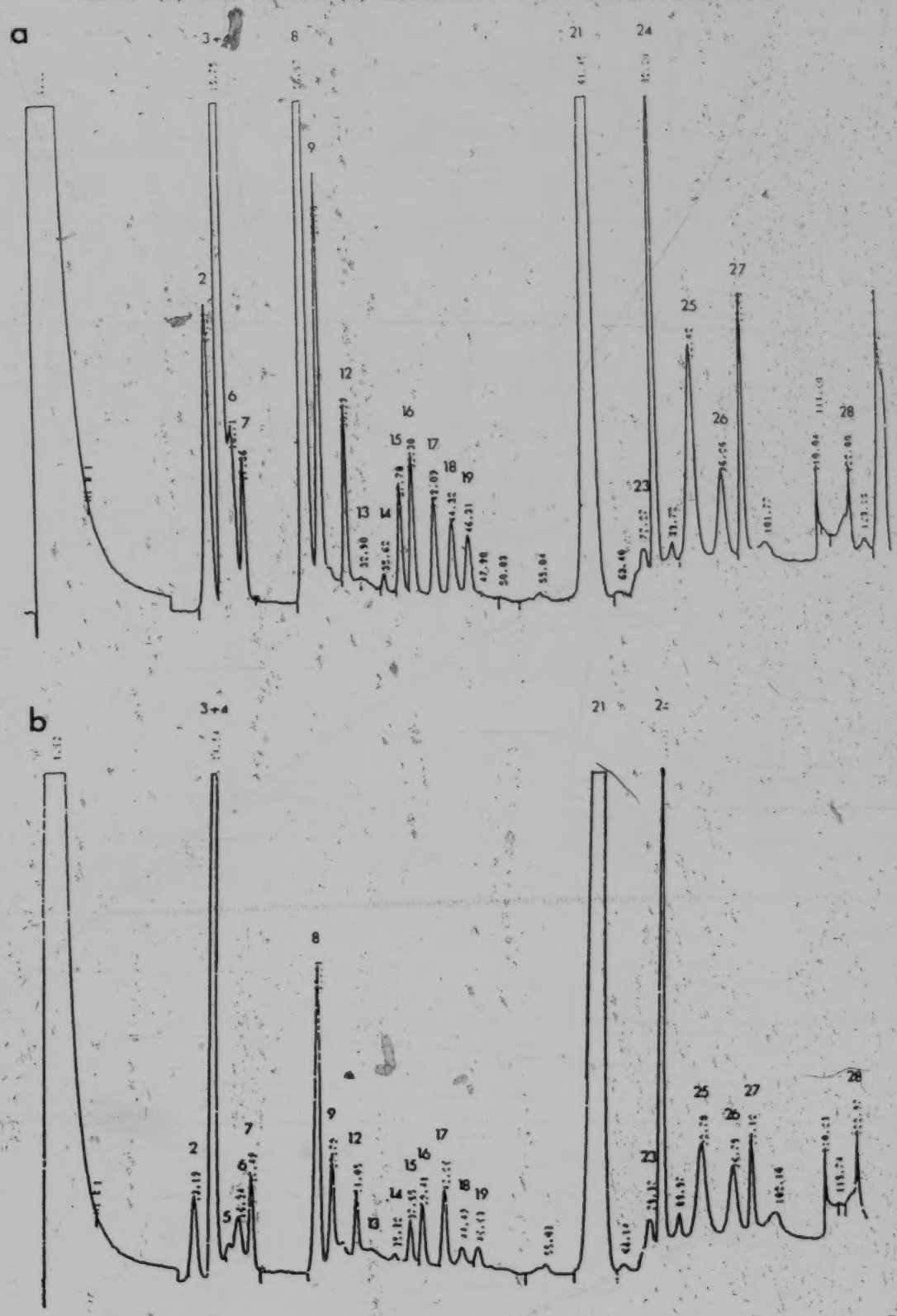


Table 3.2 **The free amino acid content (nmol/mg protein) of normal fibroblast homogenates**

Amino Acid	GM00010 N=2		Control Normal FBB, N=2		Reported[1] Normal values N=3, mean (range)
Aspartic	0.68	1.24	N/A	N/A	5.10 (2.60 - 7.00)
Threonine	N/A[3]	N/A	N/A	N/A	2.2 0(2.10 - 2.50)
Serine	N/A	N/A	N/A	N/A	2.4 0(2.10 -2.80)
Proline[2]	-	-	-	-	3.8 0(3.40 - 4.20)
Glycine	3.01	5.50	4.07	3.77	5.40 (4.50 - 7.20)
Alanine	2.20	4.20	4.42	4.95	4.00(3.3 0- 4.90)
Valine	1.10	1.50	1.55	1.55	1.50 (1.10 - 2.10)
1/2 Cystine	**0.53**	**0.63**	**0.67**	**0.73**	**NM[4]**
Methionine	0.24	0.50	0.55	0.51	2.60(0.70 - 4.60)
Isoleucine	0.88	1.40	1.37	1.38	0.80 (0.62 - 1.10)
Leucine	1.10	1.70	1.83	1.89	2.00 (1.70 - 2.50)
Tyrosine	0.66	1.10	1.08	1.20	1.40(1.20 - 1.60)
Phenylalanine	0.64	1.00	0.96	1.04	0.63 (0.42 -0.90)
Lysine	1.20	1.45	1.50	1.82	1.75 (1.50 - 2.00)
Histidine	0.73	1.00	0.96	1.10	1.80 (1.50 - 2.00)
Arginine	1.40	1.76	1.66	2.34	4.00 (3.50 - 4.40)

[1] Tietze and Butler, 1979

[2] Proline and hydroxyproline are not detected by this system

[3] N/A results not available due to co-elution

[4] NM not measurable at the instrumental sensitivity

Table 3.3 **The free amino acid content (nmol/mg protein) of Cystinotic fibroblast homogenates**

Amino acid	Cystinotic FBB GM00090A[1]		Cystinotic FBB #1324	Cystinotic FBB #717
Glycine	1.50	1.15	2.40	2.50
Alanine	3.20	3.60	2.74	2.68
Valine	1.44	1.84	0.96	1.17
1/2 Cystine	**5.40**	**7.60**	**2.80**	**2.30**
Methionine	0.34	0.43	0.30	0.35
Isoleucine	1.10	1.53	0.87	1.18
Leucine	1.40	1.94	1.33	1.70
Tyrosine	0.92	1.22	0.70	0.76
Phenylalanine	0.93	1.16	0.64	0.76
Lysine	1.34	1.60	1.10	1.40
Histidine	0.97	1.07	0.81	1.08
Arginine	1.80	2.02	1.10	1.51

[1] The data represent two sets of measurements for cystinotic cell line GM00090A

Table 3.4 **The free amino acid content[1] (nmol/mg protein) of I-cell disease fibroblast homogenates**

Amino acid	I-cell FBB (#454)	I-cell FBB (GM02013B)	I-cell FBB (GM013211F)
Glycine	1.94	1.49	5.15
Alanine	3.80	1.90	7.67
Valine	1.68	1.93	1.88
1/2 Cystine	**0.80**	**1.01**	**N/D**
Methionine	0.43	0.60	0.59
Isoleucine	1.46	1.68	1.56
Leucine	1.65	2.16	1.88
Tyrosine	1.00	1.18	1.30
Phenylalanine	1.00	1.13	1.15
Lysine	1.39	2.00	1.93
Histidine	0.97	0.90	1.31
Arginine	1.54	1.72	1.99

[1] The values represent one set of measurement for each cell line

Table 3.5 **Comparison of free amino acid content (nmol/mg protein) of high density lysosomal fractions of normal (GM00010) and cystinotic (GM00090A) fibroblasts**

Amino acid	Normal GM00010 N= 3 mean ± SD	Cystinotic GM00090A N= 3 mean ± SD
Glycine	90.20±37.3	48.38±25.2
Alanine	9.40±3.6	8.82±5.82
Valine	2.74±1.95	3.28±0.11
1/2 Cystine	**N/D**[1]	**9.30±0.7**
Methionine	0.40±0.35	0.45±0.02
Isoleucine	1.81±1.14	2.00±0.57
Leucine	2.30±1.35	2.73±0.33
Tyrosine	1.90±1.87	2.11±0.42
Phenylalanine	1.40±1.30	1.31±0.30
Lysine	3.50±1.53	3.00±0.71
Histidine	3.13±1.80	2.65±0.64
Arginine	1.60±0.50	1.46±0.91

[1] N/D non detectable

Table 3.6 **Ratio of cystine content in relation to valine, leucine, and lysine content in cystinotic fibroblast (line# 1324) homogenate and high density lysosomal fractions.**

Amino acid ratio	Cystinotic homogenate	Cystinotic lysosome (no wash)	Cystinotic lysosome (wash 1)	Cystinotic lysosome (wash 3)
cystine / valine	2.92	1.12	1.92	4.50
cystine / leucine	2.10	1.30	2.47	3.36
cystine / lysine	2.54	1.23	1.77	1.91

Table 3.7 **The effect of the washing procedure on the free amino acid content (nmol/mL sample) in high density lysosomal fractions of I-cell disease fibroblasts (GM02013B)**

Amino acid	Lysosome (no wash)	Lysosome (wash 1)	Lysosome (wash 3)
Glycine	12.9	4.5	13.1
Alanine	1.53	0.4	0.9
Valine	0.92	0.22	0.59
1/2 Cystine	**N/D**	**N/D**	**trace**
Methionine	0.18	0.06	0.07
Isoleucine	0.58	0.16	0.32
Leucine	0.78	0.23	0.44
Tyrosine	0.60	0.14	0.29
Phenylalanine	0.49	0.13	0.24
Lysine	1.08	0.18	0.67
Histidine	0.81	0.31	0.59
Arginine	0.93	0.17	0.58

Chapter four: Discussion

To date, there have been no published reports on the total free amino acid content of human fibroblast lysosomes. However, the intralysosomal amino acid pool has been studied in rat liver lysosomes (Harms et al, 1981). This work is an attempt to develop methods to investigate the intralysosomal amino acid content of normal and mutant human fibroblasts. Cystinotic and I-cell disease fibroblasts were primarily selected to test the methodology as in both these disorders, there is an expected intralysosomal accumulation of cystine due to defective carrier mechanism for transport of cystine (Tietze et al, 1986). A secondary aim was to document the lysosomal amino acid profiles in both disorders, since it is possible that other amino acid transport mechanisms might be defective.

1. Tissue culture

In agreement with previous reports (Greene et al, 1985), sensitivity of I-cell fibroblasts to freezing, was observed. The lack of neuraminidase enzyme activity in this disorder leads to an increase in the quantity of sialic acid-containing residues which seems to confer this increased sensitivity to freezing (Sly et al, 1976).

2. Subcellular fractionation of human fibroblasts and marker enzyme assays

The isolation of lysosomes has always been a challenge to investigators, because of the fragility of these organelles, their physiological heterogeneity, and the different experimental variables involved in this procedure.

Isolation of lysosomes is best judged by careful quantitation of the distribution of marker enzyme activities. Subcellular fractionation of fibroblasts on Percoll gradient resulted in a bimodal distribution of lysosomal enzyme (β-hexosaminidase) activity. The first peak is a low density peak which occurs in the region of the gradient where endosomes, Golgi apparatus, endoplasmic reticulum, and plasma membrane fractionate. The second peak is a high density peak which typically contains the majority of the lysosomal marker enzyme activity. The ratio of activities present in the two peaks was found to vary. This variation is probably due to the physiological state of the lysosomal compartment and is a reflection of the biosynthetic origin of lysosomes and their involvement in different endocytic processes. In this study of intralysosomal amino acid content the high density lysosomal fractions were emphasized since they appear to be purer fractions. The purity of the lysosomal fractions was assessed by the absence of marker enzyme activities of other organelles, and by electron microscopical study of the granular fractions and the heavy lysosomal fractions were found relatively pure.

In I-cell disease, newly synthesized lysosomal hydrolases are secreted in the extracellular medium instead of being targeted correctly to lysosomes, therefore, there is a characteristic pattern of lysosomal enzyme deficiencies. In agreement with previous reports, the results of this study showed that β-hexosaminidase activity in I-cell fibroblast homogenate was between 10 - 17% of that in the normal fibroblast homogenate i.e. results of this study (6.86 and 1.18 μmol / hr / mg protein in the normal and I-cell fibroblast homogenates, respectively), agreed with reported ranges (6.49 - 10.77 and 0.83 - 2.04 μmol / hr / mg protein in the normal and I-cell fibroblast homogenates, respectively, Tietze and Butler, 1979).

3. Electron microscopy

"Nowadays, when an electron microscopist sees a membrane-bound body, containing dense deposits, membranous whorls, or other materials with a messy appearance, he knows that he is most likely looking at a lysosome".

(de Duve, 1983)

In the electron micrographs of lysosomal specimens, numerous heterogenous cytoplasmic vacuoles with single membranes, which can be considered lysosomes, are seen. They are clearly distinct from mitochondria and from small vesicles with crystalline inclusions (peroxisomes) which can be identified by their unique and uniform ultrastructure. Therefore electron microscopical study was helpful in assessing the purity of the granular fractions. However, it was suggested that lysosomes can only be conclusively identified as such by means of enzyme staining (Schellens et al, 1977). Acid phosphatase was originally the most commonly used enzyme; but recently the activity of several other hydrolases have been demonstrated at both the light and electron microscope levels. Studies have shown that it is difficult to obtain specific enzyme substrates in order to locate the enzymes unambiguously because other enzymes may also transform the same substrates. More recent reports have used immunocytochemical techniques as an alternative means to accurately identify lysosomes. Antibodies specific to lysosomal enzymes, proteins and characteristic membrane antigens can be obtained. Electron absorbing materials can be linked to the antibodies and the combination detected by electron microscopy (Holtzman, 1989).

4. Amino acid analysis

Ion exchange analysis has been the traditional methodology of quantitation of free amino acids present in biological fluids and tissues, and it has proved to be an important tool in the diagnosis of various genetic diseases.

4.1 Preparation of the samples for amino acid analysis

It was found in the preliminary trials of amino acid analysis that the free amino acid content of lysosomal fractions was very low and some form of concentration of the samples was necessary. Reduction in the volume of Percoll gradient used in the fractionation resulted in a significant improvement in the recovery of amino acids.

One of the major concerns encountered when preparing the samples for amino acid analysis, was how to wash the lysosomal fractions to avoid measuring the amino acids surrounding the lysosomes. Lysosomal fractions were washed by means of ultrafiltration through membrane cones combined with several washings with phosphate buffered saline. The findings of the washing procedure were expected to demonstrate that the amino acid content of the non washed samples should approximately equal the amino acid content of wash 1 (first filtrate) which should represent two components amino acids in the matrix outside the lysosomes and amino acids released from disrupted lysosomes, plus that of wash 3 (final filtrate) which represents the intralysosomal content. This was the case in some samples but other results showed a much lower amino acid content in the washed samples indicating that losses must have occured during the washing procedure. Therefore, amino acid content in both the washed and non washed samples is presented as raw data.

Studies have shown that storing plasma samples at -20°C for seven days before deproteinization led to marked reduction in the concentrations of disulfide amino acids (Deyl et al, 1986). This was reaffirmed in this investigation: The immediate acidification of the samples with sulfosalicylic acid before storing was found to improve the recovery of cystine. Therefore, it is advisable to remove proteins as soon as possible and to store the deproteinized samples at - 70°C.

4.2 Problems encountered in amino acid analysis

In this study, amino acids were separated on a cation exchange column with continuous post column derivatization with OPA. The advantages of using OPA-derivatives for amino acid detection are: a) OPA-derivatives exhibit high fluorescent yield, b) they are soluble in aqueous buffer, c) the derivatization reaction occurs quickly, and d) OPA is not fluorescent, itself interfering with the reaction. However, several problems were encountered. Firstly, was the inability to measure secondary amino acids (proline and

hydroxyproline) as OPA does not form fluophors with secondary amine groups. It has been shown that detection of secondary amino acids with OPA is possible in the presence of sodium hypochlorite (Deyl et al, 1986). However this was not attempted since it was reported that sodium hypochlorite diminishes the response of many primary amino acids (Mc Clung and Frankenberger jr, 1988), and quantitation of these secondary amino acids was not crucial for the success of the project.

Secondly, shifting in retention times of the more acidic amino acids occured at the start of the runs. This group of amino acids also co-eluted forming large peaks. Running reagent blanks with amino acid calibration standard showed the same finding indicating that this shift could be due to the pH , or the composition of the medium, or the preparation technique of the samples. In this study, the samples were acidified with sulfosalicylic acid which is known as the most suitable deproteinization reagent (Deyl et al, 1986). When the pH of deproteinized samples was tested before injection into the analyzer, it was found < 2 which is almost ideal for subsequent ion-exchange chromatography. With regard to ultrafiltration of the samples as a step in the preparation technique, studies showed that this seem to decrease the retention times during chromatographic separation on ion exchange columns, leading consequently to distorted separation of critical pairs of amino acids such as serine-threonine, asparagine-glutamine, and tyrosine-phenylalanine. Resolution could presumably be improved by altering conditions, but this was not attempted in the scope of this study.

Thirdly, large peaks of GABA occurred in filtrates of lysosomal fractions compared with homogenate samples. GABA is known to be physiologically abundant in the central nervous system, kidney, and intestines. In body fluids, particularly high levels are seen in cerebrospinal fluid in psychiatric disorders, and in hyper-β-alaninaemia. Increased levels are also found after 6-azauridine treatment (Deyl et al, 1986). Reagent blanks were run to try to identify a possible source of contamination resulting in this increased GABA content. A peak eluted at the same retention time as GABA, only in the Percoll blank chromatogram. This might explain the larger GABA peak seen in the chromatogram of the lysosomal fractions in comparison with the homogenate samples which were not put on Percoll gradient. However, questions are raised as to the identity of this peak? Is it GABA? Is it a contaminant eluting at the same retention time as GABA? If it is a contaminant what is it? and how could one get rid of it? These questions are yet to be answered with further investigations as no mention of this finding has been reported in the literature.

Glycine eluted in huge peaks, especially, in lysosomal samples compared to homogenate samples. Contaminants derived from sources such as dust, solvents, water, paper, glassware, and cornstarch from gloves can present background levels of amino

acids such as serine, glycine, and glutamic acid (Cohen and Strydom, 1988). Therefore the extensive manipulation involved in isolating lysosomal fractions could explain the increase of glycine in the fractions compared with the crude homogenate. In this study, reagent blanks showed background amino acids such as glutamic acid, glycine, traces of ornithine, OH-lysine, histidine, arginine, and other contaminants such as urea and ammonia. The huge hydroxy-lysine peaks eluting in lysosomal fractions might be partly due to background contamination.

4.3 Free-Amino acid content in normal, cystinotic, and I-cell fibroblast homogenates

The free amino acid content of the control and mutant fibroblast homogenates was found to be consistent with previous reports (Tietze and Butler, 1979), except for histidine and arginine which had lower values, and secondary amino acids (proline and hydroxy-proline) which were not measured by the OPA detection system.

In early studies performed in nephropathic cystinosis patients, cystine level was reported to be increased a hundred fold in cystinotic fibroblasts compared to normal cell lines i.e. < 0.2 and 6.6 - 10.6 nmol 1/2 cystine / mg protein in normal and cystinotic fibroblasts respectively (Schneider et al, 1967). More recently, the range of cystine levels in cystinosis has been described as 10 to 1000 times the normal levels (Gahl et al, 1989). Other reports showed a higher range of free 1/2 cystine content in normal fibroblast homogenates i.e. 0.0 - 0.57 nmol / 1/2 cystine / mg protein (Tietze and Butler, 1979). The increase in cystine levels in cystinotic fibroblasts was successfully demonstrated by this methodology: a thirteen fold increase in one cell line (GM00090A) and about five fold increase in the other cell lines (Lines# 1324, 717). It is important to note though that the cystine peak eluting in the control fibroblast samples seemed broader and unlike that eluting in the calibration standard. Therefore, this peak either may or may not be cystine and in the latter case the finding of this investigation would be more in line with the one hundred fold increase in cystine levels reported earlier in cystinotic lines compared to normal ones.

Three clinical varieties of cystinosis have been described in the literature (Gahl et al, 1989). The most common group is infantile nephropathic cystinosis with an early onset and rapid progression to renal failure in the first decade of life. In intermediate (adolescent) cystinosis, patients manifest kidney disease at a later onset and have a variable progression of renal malfunction. Benign (adult) cystinosis patients have no renal involvement at all and live normally as adults except for having corneal crystals. In addition to their milder disease compared with classical nephropathic cystinosis, patients with cystinosis variants store lesser amounts of cystine within their cells. The three cystinotic cell lines used in this investigation were all from patients with typical infantile nephropathic cystinosis, but

showed a wide range of cystine content. The explanation for this is not entirely clear, but considerable variation in cystine content in cystinotic cell lines has been also reported by others and was thought to occur as a result of experimental variables such as storage temperature, method of fibroblast detachment, age and passage number of cells, and composition of culture medium.

When comparing methods for measuring cystine content, Oshima et al (1974) showed that as little as 10 picomole of cystine could be measured in cultured skin fibroblasts from patients with nephropathic cystinosis, by the cystine binding protein assay. Because the aim of this research is to study complete amino acid profiles, the use of the protein binding assay was not considered. On the other hand, the HPLC system used in this study is ten times more sensitive than the protein assay (an analytical detection limit as low as 1 picomole for each individual amino acid).

The free amino acid content of I-cell fibroblasts was found consistent with previous reported ranges (Tietze and Butler, 1979), but more towards the lower values. The cystine content of the I-cell disease was only slightly higher than controls. Previous studies showed that cystine in I-cell lines can be in the same range seen in cystinotics. However, on the other hand, low levels or virtual absence of cystine have been reported in other laboratories (Greene et al, 1985). This was explained to be due to the heterogeneity of the clinical picture of this disorder. It is also logical to expect that the same experimental variables mentioned above could influence the recovery of cystine from I-cell fibroblasts.

The increase of cystine in cystinotic and I-cell fibroblast homogenates was unique and was not associated with significant changes in other amino acids. It can be concluded that it is likely that there are no other major amino acid transport defects occurring in these disorders.

4.4 Free amino acid content of high density lysosomal fractions of normal, cystinotic, and I-cell fibroblasts

Intralysosomal amino acid content varied considerably from one sample to the other in one cell line and among cell lines. However, it was possible to successfully demonstrate the presence of high cystine levels in cystinotic lysosomal fractions in comparison with controls.

Cystine levels in granular fractions of cystinotic fibroblasts were shown to be four to five fold higher than the crude homogenate (Harms and Schneider, 1979). Findings of this study showed approximately two - four fold enrichment of cystine in the cystinotic lysosomal fractions compared to the crude homogenate.

The I-cell lysosomal fractions showed only traces of cystine, which was consistent with the finding of relatively low cystine levels in the I-cell homogenate samples. The occurrence of such finding was reported in other laboratories and it was suggested to be due to the heterogeneity in clinical and biochemical features of this disease (Tietze and Butler, 1979). Cystine content of I-cell fibroblasts was found to increase with subculture and with age of cell lines (Crawhall et al, 1977).

The large variations that were found in presumed intralysosomal amino acid concentrations in this study were rather disappointing. They might be explained by the numerous experimental variables involved in the isolation methods, the physiological heterogeneity of lysosomes, and in case of the mutant cell lines, the clinical heterogeneity. also the protein content, used as denominator, may have contributed to the variability, particularly in I-cell fibroblasts which are known to be morphologically abnormal. Therefore, one wonders if it is difficult to define the intralysosomal amino acid pool due to such heterogeneities. A larger number of assays provide a more accurate assessment of expected intralysosomal amino acid concentrations.

The objective of this study was to develop a method to screen candidate genetic diseases for lysosomal amino acid transport defects. Progress have been made towards the objective, but the variability in results limits the potential utility of the method. A further approach that might address that problem would be to standardize more the culture conditions, especially passage number, confluency, and consistency of the growth medium prior to harvesting.

The method could be modified to incorporate loading of lysosomes by adding methyl esters of amino acids of interest to the culture medium. This would improve the sensitivity of the method, as well as possibly revealing more subtle defects in amino acid transport. Also, it would be possible by amino acid profiling of incubated lysosomes over time to obtain more direct data on egress rates of several amino acids simultaneously in various conditions.

Conclusions

The attempt of developing methods to measure amino acid concentrations in lysosomes of normal and mutant human fibroblasts lead to the following conclusions:

1- Mutant fibroblasts (cystinotic and I-cell disease) grow at a much slower rate than normal cell lines. I-cell disease fibroblasts are sensitive to freezing.

2- Mutant fibroblasts require more gentle homogenization than normal ones to avoid disrupting lysosomes.

3- Percoll forms a blue color with the Folin reagent, therefore its interference should be corrected for when quantitating proteins in gradient fractions.

4- Percoll can be eleminated from lysosomal fractions by washing in buffers together with ultrafiltration. However, this affects the recovery of amino acids and results in some losses due to the fragility of the lysosomes.

5- To ensure a good recovery of amino acids in general and to a greater extent cystine, samples must be immediately acidified with solid sulfosalicylic acid and stored in -70°C.

6- One of the major drawbacks encountered in amino acid analysis of the fibroblast homogenates and lysosomal fractions with ion exchange chromatography, was shifting of retention times with incomplete resolution of acidic amino acids at the start of the run. This problem needs to be further investigated and alteration of conditions such as pH, composition of suspension buffers, and sample preparation, needs to be made.

7- The GABA peaks which eluted in the lysosomal fractions were much larger compared with those in fibroblast homogenate samples due to contamination from Percoll. Glycine and hydroxylysine peaks were also significantly larger in the lysosomal fractions compared with fibroblast homogenate samples. This occurs partly due to contamination from dust, glassware, corn starch from gloves, deionized water, and reagents. However, further investigations need to be carried out to identify the nature of the peak eluting at the retention time of GABA.

8- Amino acid concentrations in normal and mutant fibroblast homogenates were fairly consistent and reproducible. The method demonstrated successfully a five to thirteen fold increase in cystine in cystinotic fibroblasts compared to normal ones. Cystine content of I-cell disease fibroblasts varied from non detectable to slightly higher than in normal ones.

9- Cystinotic and I-cell disease fibroblasts did not show an unusual pattern of amino acid concentrations, apart from increased cystine suggesting that there are no other defective amino acid carriers.

10- Cystinotic lysosomal fractions had a significantly high level of cystine compared to the normal fractions; while I-cell disease fractions had only traces of cystine.

11- Intralysosomal amino acid concentrations showed considerable variations which may be due to experimental variables, physiological heterogeneity of lysosomes, and in case of mutant cell lines, heterogeneity of the clinical disorders.

12- The potential utility of the method is rather limited due to the variability of the results. Standardization of some experimental variables need to be attempted: i.e.cell passage number, confluency, and consistency of the growth medium prior to harvesting.

13- The method could be modified to incorporate loading of lysosomes with methyl esters of amino acids of interest to improve its sensitivity and to reveal subtle defects in amino acid transport.

14- Other candidate genetic diseases could be screened by the method with the use of the appropriate technique of detection of the abnormally stored material.

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